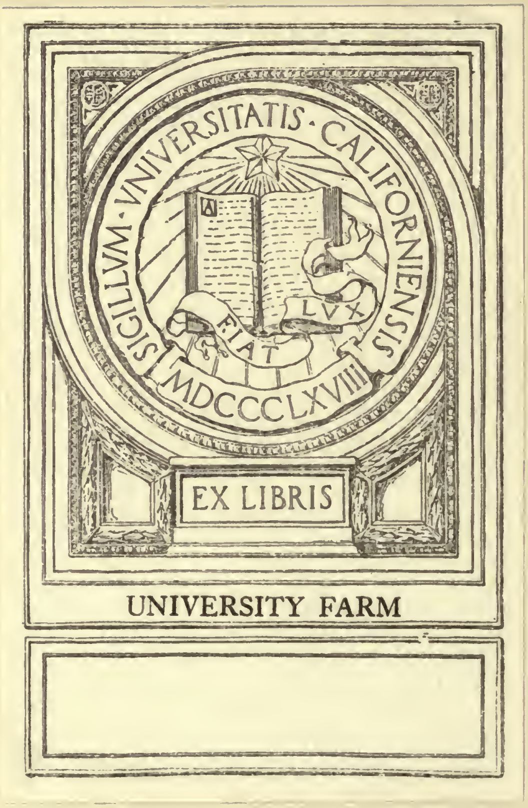
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MEDICAL BACTERIOLOGY

RODDY

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BY

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PREFACE

The more intimate one's knowledge of general bacteriology and its special subdivisions, the better is he equipped to engage in any particular branch of the work. Some familiarity with physics and chemistry is essential and the extent of one's knowledge in these allied sciences largely determines the extent to which one can cope with bacteriological problems.

This subject has so expanded that a complete systematic exposition of it is no longer possible in a single volume, and the frequency with which valuable new facts are disclosed, and new tests and technique devised makes it imperative for one engaged exclusively in bacteriological work to read the current journals devoted to it.

The application of bacteriology to the solution of many important problems arising in medicine, veterinary surgery, agriculture and industry has created a demand that some knowledge of this science be possessed by those engaged in diverse occupations.

There are basic facts and technique common to all branches of bacteriology and in each specialty a few procedures of prime importance. A knowledge of these is the first requirement of all beginners; this alone constitutes the most valuable information on the subject that can be imparted in medical and other general courses of instruction. It is also the foundation necessary for those who intend to devote themselves exclusively to bacteriology, and for further independent work in this field.

The author, being cognizant of the needs of students, practitioners of medicine, pharmacists and those engaged in the foodstuff industries, and having the invaluable advice and guidance of Prof. R. C. Rosenberger and the able assistance of Dr. Louis Gershenfeld, has endeavored to present in the clearest form a text book for beginners and laboratory guide for medical practitioners and pharmacists.

It is a pleasure to acknowledge the invaluable assistance rendered by Dr. Robert M. Lukens, who made the illustrations, Mr. David R. Brewer, Dr. M. E. Smoczynski and Mrs. Mary L. Vogel.

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CONTENTS

PART I

CHAPTER I

PAGE
Introduction.......... 1

CHAPTER II

Classification of Bacteria 4

CHAPTER III

Microscopy.......... 9

CHAPTER IV

Staining 15

CHAPTER V

Sterilization 23

CHAPTER VI

Culture Media 40

CHAPTER VII

Staphylococci.......... 55

CHAPTER VIII

Streptococci 59

CHAPTER IX

Pneumococcus (Diplococcus Lanceolatus) 63

CHAPTER X

Meningococcus 68

CHAPTER XI

Gonococcus 71

CHAPTER XII

PAGE
Micrococcus Catarrhalis 74

CHAPTER XIII

Micrococcus Tetragenus and Sarcina. 75

CHAPTER XIV

Bacillus of Influenza, Koch Week's Bacillus, and Bordet-Gengou Bacillus 77

CHAPTER XV

Bacillus of Morax and Axenfeld 79

CHAPTER XVI

Pneumobacillus or Bacillus of Friedländer (Bacillus Mucosus Capsulatus) 80

CHAPTER XVII

Diphtheria Bacillus (Klebs-Loeffler Bacillus) 82

CHAPTER XVIII

Pseudo-Diphtheria Bacillus; Hoffman's Bacillus; Xerosis Bacillus.. 89

CHAPTER XIX

Bacillus and Spirillum of Vincent 91

CHAPTER XX

Tubercle Bacillus 94

CHAPTER XXI

Bacillus of Leprosy, The Smegma and Other Acid-Fast Bacteria 101

CHAPTER XXII

The Colon Bacillus 103

CHAPTER XXIII

The Typhoid Bacillus 106

CHAPTER XXIV

The Paratyphoid and Similar Bacilli. 109

CHAPTER XXV

Diagnosis of Typhoid Fever and Food Poisoning Infections. 111

CHAPTER XXVI

PAGE
Dysentery Bacilli 119

CHAPTER XXVII

Spirillum Choleræ Asiaticæ, Vibrio Choleræ Asiaticæ (Comma Bacillus). 122

CHAPTER XXVIII

Micrococcus Melitensis 125

CHAPTER XXIX

Bacillus Pyocyaneus 126

CHAPTER XXX

Bacillus Proteus Vulgaris 128

CHAPTER XXXI

Bacillus Lactis Aerogenes, Bacillus Bulgaricus 129

CHAPTER XXXII

Bacillus Botulinus. 130

CHAPTER XXXIII

Bacillus Aerogenes Capsulatus 132

CHAPTER XXXIV

Bacillus Maligni Œdematis. 134

CHAPTER XXXV

Bacillus Tetani 136

CHAPTER XXXVI

Bacillus Anthracis. 139

CHAPTER XXXVII

Bacillus Subtilis. 141

CHAPTER XXXVIII

Bacillus Prodigiosus 143

CHAPTER XXXIX

Bacillus Pestis 144

CHAPTER XL

PAGE
Bacillus Mallei 147

CHAPTER XLI

Spirochæta, Obermeyeri 149

CHAPTER XLII

Treponema Pallidum 150

CHAPTER XLIII

Spirochæta Pertenuis 154

CHAPTER XLIV

The Higher Bacteria. 155

CHAPTER XLV

Hyphomycetes (Molds) 161

CHAPTER XLVI

Saccharomycetes 164

CHAPTER XLVII

Monila 167

CHAPTER XLVIII

Sporotrichum Schenkii 168

CHAPTER XLIX

Infectious Diseases of Unknown Causation 169

PART II

CHAPTER I

Examination of Water 173

CHAPTER II

Examination of Milk 176

CHAPTER III

PAGE
Examination of Fluids and Solids 178

CHAPTER IV

Determination of the Germicidal Power of Chemical Disinfectants. 185

CHAPTER V

Diagnosis 201

CHAPTER VI

Bacterial Vaccines. 211

CHAPTER VII

Therapeutic Sera 223

CHAPTER VIII

Wassermann and Other Complement Fixation Tests 226

CHAPTER IX

Immunity. 257
Index 279

MEDICAL BACTERIOLOGY

PART I

CHAPTER I

The first intimation that disease might be due to minute organisms was given by Kircher, a member of the Society of Jesus, in 1646. He reported the presence of "minute living worms" in putrid meat, attributed the putrefaction to their activity and suggested that disease might be due to similar organisms.

Twenty-five years later he reached the conclusion that puerperal fever was caused by animalculæ (bacteria). In 1658 Hook made similar observations and confirmed Kircher's work.

Leeuwenhoek, a linen draper, who entered his trade in childhood and lacked schooling, studied lens making, devised lenses much superior to any previously made, and so opened up vast possibilities. With his improved instruments, he observed animalculæ in saliva, feces and vegetable infusions. His observations were reported in 1660.

At that time the discoveries of these men excited some interest, but it was not general and advancement lagged for more than a half a century. Then in 1762 Plenciz confirmed the observations of Kircher and Leeuwenhoek and assumed a relationship between animalcules and disease in general.

Spallanzani, in 1777, demonstrated that boiling and hermetically sealing infusions prevented fermentation.

Microscopic observations were becoming more scientific and in 1786 Muller divided animalculæ into two classes—monas and vibrio.

At the close of the eighteenth century (1798) another great advance was made when Jenner discovered the method of immunizing against small pox, a method that is still in vogue.

In 1835 Bassi reported certain diseases of silkworms due to the activity of fungi. In 1840 Henle postulated that which is commonly referred to as "Koch's Law:"

I. A specific microörganism must be constantly associated with the disease

II. It must be isolated and studied apart from the disease.

III. When introduced into healthy animals it must produce the disease, and in the animal in which the disease has been produced experimentally, the organisms must be found under the original conditions.

Semmelweiss, of Vienna, a brilliant martyr, sacrificed by the bigotry of his profession, in 1847 reached the conclusion that the cause of puerperal fever was transmitted from the dissecting room to patients by students' fingers, and to curtail its occurrence advised students to wash their hands prior to making vaginal examinations. For this innovation the elect ostracized him.

In 1850 Rayer and Davine discovered a bacterium in the blood of animals afflicted with splenic fever. Thirteen years later Davine disclosed the relation of bacillus anthrax to splenic fever.

Until this time microörganisms associated with putrefaction and disease had been classified as worms or animal organisms; Robin, in 1853, classified them with the genus leptothrix, as belonging to the algæ. In 1859 Davine studied the subject in a broader way than had been done before and insisted upon their vegetable nature. Later Colm confirmed these contentions and bacteria have since been generally considered as vegetable organisms.

By the middle of the nineteenth century the belief in a relationship of bacteria to disease was beginning to affect medical thought, as evidenced in Henle's "Text Book of Rational Pathology," published in 1853. Reinfleisch declared wound infection was due to microbic invasion. In 1866, two years later, Walder expressed the same belief.

Regardless of the many observations, the collection of considerable evidence and the presentation of many hypotheses, some of which were surprisingly accurate, the true relationship of bacteria to life and disease was not appreciated until after the publication, by Pasteur, in 1869, of the first complete study of a contagious disease caused by microörganisms. To this man and the country which produced him and facilitated his investigations, the world is indebted for the study and presentation of the subject in such a way as to awaken general interest, and bring forth untold blessings.

About this same time Koch introduced the cultivation of bacteria on solid culture media and in many ways greatly expanded the possibilities of bacteriological studies.

From that time on great discoveries followed rapidly, one after the other, most of them arrived at after years of study and research, few by accident.

In 1875, Lister inaugurated antiseptic surgery, an epoch in that art.

Ehrlich and Weigert introduced staining methods for the study of bacteria in 1877. In 1879 Frank began the study of the relationship of bacteria to the growth of leguminous roots.

The year 1884 may be considered the golden year of bacteriology; among other advances made may be noted the discovery of the diphtheria bacillus by Klebs and Loeffler, the pneumococcus by Fraenkel, the tetanus bacillus by Nicolaier, Gaffky's extended studies of the typhoid bacillus (discovered four years earlier by Eberth), the discovery by Pasteur of his method of immunizing against rabies, and the discovery by Koch of the spirillum of cholera.

A great stride forward was taken in 1890 when Behring introduced diphtheria antitoxin.

The discovery of the specific cause of syphilis by Schaudin in 1905, the introduction of opsonic therapy by Wright in 1907, and the antimeningococcus serum of Flexner, are but a few of the recent advances in this rapidly developing science.

The most notable recent event in the history of American bacteriology was the publication of the first number of the Journal of Bacteriology, January, 1916, the first American journal devoted exclusively to this science.

A more complete and interesting presentation of the history of bacteriology may be found in the following books:

"Vaccination, Its Natural History and Pathology," S. M. Copeman.

"History of The Plague in London," Daniel DeFoe.

"Infection and Parasitic Diseases Including Their Causes and Manner of Transmission," Millard Langfeld.

"Bacteria," George Newman.

"Scientific Features of Modern Medicine," Frederic S. Lee.

"History of Medicine," Fielding H. Garrison.

"History of Medicine," J. H. Baas, translated by Handerson.

CHAPTER II

CLASSIFICATION

Bacteria have some properties allied to the lower forms of animal life—flagellata—but their closest relationship is to the lower algæ. They are generally considered to be vegetable organisms and are classified as fission fungi or **schizomycetes.** They are microscopic, unicellular organisms which propagate by cleavage or fission. Of the various systems of classification of bacteria, none of which is complete or satisfactory, the one most commonly adopted by writers is the following by Migula.

CLASSIFICATION OF BACTERIA

Coccaceæ.—Spherical forms, with divisions in one to three planes, forming two to eight daughter cells.

- **Streptococcus.**—Cocci without flagella, dividing in one plane, often remaining connected so as to form beaded chains.
- **Micrococcus.**—Without flagella dividing in two planes. When remaining connected, after fission, they form grape-like clusters (staphylococci).
- **Sarcina.**—Without flagella, dividing in three planes. Usually remaining connected to form cubical packets.
- **Planococcus.**—Possessing flagella, dividing in two planes, sometimes remaining connected to form sheets.
- **Planosarcina.**—Possessing flagella, dividing in three planes,

Bacteriaceæ.—Elongated straight cells dividing in the transverse plane only.

- **Bacterium.**—Without flagella, sometimes with endospores.
- **Bacillus.**—With flagella and sometimes endospores.
- **Pseudomonas.**—With polar flagella, seldom with endospores.

Spirillaceæ.—Cells forming spirals or parts of spirals, dividing in the transverse plane only.

- **Spirosoma.**—Inflexible cells without organs of locomotion.
- **Microspira.**—Inflexible cells with from one to three polar flagella.
- **Spirillum.**—Inflexible cells with from five to twenty curved polar flagella, forming tufts.
- **Spirochœta.**—Flexible undulating cells without flagella, but possibly a lateral undulating membrane.

Chlamydobacteriaceæ.—Cells of different forms, united in branches or unbranched chains and provided with capsules or envelope.

- **Streptothrix.**—Elongated cells in non-branching chains, divisions in one plane only; reproducing by non-motile-conidia.
- **Cladothrix.**—Branching chains, dividing in one plane only. Motile reproductive cells (Zoogonidia) with polar flagella.

Crenothrix.—Unbranched chains, dividing in three planes, forming rounded daughter cells.

Phragmidiothrix.—Cells first unbranched, with very delicate sheath, dividing in three planes and sometimes branching later.

Thiothrix.—Unbranched, non-motile threads, containing sulphur granules, dividing in one plane only.

Beggiatoaceæ.—Cells without capsules, joined in unbranched threads; provided with an undulating membrane.

Beggiatoa.—Cells with the characteristics of the family, containing sulphur granules.

Orla Jensen's classification is based upon the nutritive characteristics. In this the bacteria are grouped according as they are: (1) autotrophic, *i.e.*, able to form protein and carbohydrates from inorganic carbon and nitrogen; (2) semi-autotrophic, *i.e.*, require organic carbon compounds, but able to form protein from inorganic nitrogen; or (3) heterotrophic, *i.e.*, requiring for nourishment organic compounds of both carbon and nitrogen.

There are excellent reasons for accepting the suggestion of Victor Vaughan, who believes it expedient to consider bacteria as such, and to classify them as a separate group of living organisms, and not as a branch of either the vegetable or animal kingdoms.

Some bacteria are motile, and progress either by an undulatory movement, a screw-like rotary movement, or as darts; the latter possess extremely fine, long thread-like processes, called **flagella**; these are believed to be the organs of locomotion.

"Structurally, bacteria consist of protoplasm, which at the periphery is condensed to form a layer resembling a cell wall. While a nucleus has not been demonstrated, it is probable that practically the whole cell is composed of nuclear matter, there being but a thin peripheral layer of perinuclear protoplasm" (Coplin).

Some bacteria are surrounded by a **capsule.**

The chemical composition of bacteria varies for different organisms and is somewhat dependent upon the nutritive substances upon which they live. From 75 to 85 per cent. is water, 5 to 10 per cent. protein, 5 to 25 per cent. fats and 1 to 2 per cent. ash; chlorophyl is not found.

When environment becomes adverse to further development, some bacteria can transform into **spores.** Briefly stated, the first sign of endospore formation is a granular appearance of the cell protoplasm; these granules increase in size, collect in one part of the cell and coalesce, forming a round, highly refractile body, larger in diameter than the bacillus and hence causing a bulging of the cell wall. The cell protoplasm gradually, and in increasing amount, concentrates in the endospore; eventually the bacillus has disappeared and in its place remains only the endospore, globular, highly refractile, containing very little water, very much more resistant to germicides than the bacillus from which it sprung. Spore formation is not a method of reproduction or propagation. It is a device to resist destruction, a process of involution.

Arthrospore formation has been described by some authorities and denied by others.

Occasionally a culture of cocci—especially staphylococci—is found which is much more resistant to the germicidal action of heat, chemicals, etc., than the average, the resistance at times being so great as to suggest the presence of spores. Stained preparations from such resistant cultures frequently show some organisms which differ from the majority of their fellows in size; they are 50 to 100 per cent. larger than normal; some of them stain normally and others are resistant to staining. These bodies are considered to be spores (arthrospores) by some.

There are a few bacteria that thrive at 0°C. (psychrophilic) and others that flourish at temperatures above 70°C., (thermophilic) but the majority develop most luxuriantly at or near the temperature of the human body. Generally, warmth, moisture and protection from direct sun rays, are required for bacterial development.

Among both saprophytic and pathogenic bacteria, there are species which flourish in an atmosphere containing oxygen, as air, and die or change into spores when placed in an atmosphere devoid of oxygen—these are called **obligate aerobic** bacteria. There are **obligate anaerobic** bacteria which thrive only in an atmosphere free from oxygen and do not propagate when exposed to air.

Some bacteria grow best in air, but can grow to a lesser degree in the absence of air or oxygen—these are called **aerobic and facultative anaerobic.** There are bacteria, however, which are **anaerobic and facultative aerobic,** and some that grow equally well, whether in an aerobic or anaerobic environment.

The life of bacilli is very variable and is influenced by environment; in this respect different species show marked variations.

In a resting, inactive, or dormant state, some species survive for months or years. In full, continuous activity, the life of a single organism is a matter of minutes, from 16 to 20 minutes for the bacillus coli communis.

According to A. Fischer, under ideal conditions, 1,600,000,000,000,000 bacilli would develop from a single organism in 24 hours. Of course, ideal conditions for propagation never exist and increase is always much less than the potential reproductive power of any organism.

Just as for the individual, there is a limit to the growth and life of colonies of bacteria, dependent upon available food, environment and an inherent tendency to involute and die in the course of time.

Most bacteria are **saprophytic.** They live on dead animal and vegetable matter and by their activity, rapidly change its character, splitting it into simpler compounds, liberating water, carbonic acid, ammonia and ptomains. Saprophytic bacteria, with a few exceptions, are unable to survive in living animal bodies and cannot directly cause disease. Indirectly they are capable of harm. Lodged upon the dead cells covering the surface of the body of a living animal, feeding upon these dead cells and splitting them into simpler compounds, some of which are irritant or toxic to adjacent living cells, they injure and impair the function of the living cells and enhance the possibility of entrance at that point of pathogenic bacteria.

When saprophytic bacteria gain access to meat, milk, ice cream, canned vegetables, etc., they sometimes produce chemical changes which result in poisoning when these articles are ingested.

Parasitic bacteria occur chiefly or exclusively upon living animal and vegetable bodies. Some are entirely beneficent, others are injurious or destructive to certain species of animals or vegetables.

Pathogenic bacteria are those which directly cause disease. Many of them have the faculty of living either as saprophytes or parasites and hence are almost ubiquitous, commonly present in air, water and soil. Others are exclusively parasitic. Indeed, the requirements of some parasitic bacteria are so exacting that their occurrence is limited to a single species of plant or animal life.

There are three groups of bacteria distinguished by their shape: **bacilli,** which are rod-shaped, **cocci,** which are spherical, and **spirilla,** curved or spiral-shaped organisms. Morphologically, each of these three groups show numerous subdivisions.

Cocci or micrococci when observed singly are spherical or nearly so and are nearly equal in size, regardless of species. Six forms are observed:

1. **Staphylococci** arranged in irregular masses, said to resemble bunches of grapes. Frequently the manipulations incident to placing them on a glass slide and staining or otherwise preparing for microscopic study, destroys the bunch of grape-like arrangement so that we see staphylococci singly, in little irregular clumps of two, three or more elements as well as in bunches.

2. **Streptococci** occur in chains. These chains may be long or short, three, four, five or more cocci in a row forming a short chain; these short chains are usually straight. The longer chains, some of which are composed of 50 or more cocci, may be straight or curved or tangled just like a piece of rope.

3. **Tetrads** are cocci that appear in groups of four.

4. **Sarcina** are cocci arranged in cubes showing four, eight, twelve or sixteen elements on each side of the cube observed.

5. **Diplococci** those arranged in pairs, the surfaces in apposition being somewhat flattened, similar to coffee beans.

6. **Diplococci** arranged in pairs, without flattening of adjacent surfaces, elements being either spherical or lancet shaped. Some of these latter are encapsulated.

Pathogenic cocci do not possess the power of locomotion. Spore formation by cocci is a mooted question; arthrospore formation may be a property of some species.

BACILLI

Bacilli as a class are rod-shaped; some are so short and plump as to appear ovoid, others are distinctly rod-shaped. They vary considerably in size. Observing the smallest, it is difficult or impossible to determine whether or not their ends are square or round, but this can be noticed when observing large bacilli.

There are long, wide bacilli having rounded ends, long wide bacilli with square ends, long slender bacilli, some with round, others with square ends, short slender and short plump bacilli, some having round and others square ends; bacilli capable of locomotion and others that are not. Some species of bacilli, but not all, have the faculty of producing spores (endospores).

SPIRILLA

Spirilla show marked variations in size just as bacilli do. They may be divided into three groups:

1. Rigid nonmotile spirals (spirosoma).
2. Rigid motile spirals (spirillum).
3. Flexible, motile spirals (spirochæta).

Spirilla propagate by cell division, cleavage being in some instances longitudinal as well as transverse. Spore formation is unknown.

The family and genus characteristics of bacteria are permanent so far as is known. Each species is a distinct entity; staphylococci never become gonococci, colon bacilli never become anthrax bacilli, tubercle bacilli are always tubercle bacilli, they never produce anything but tubercle bacilli; mutation from one form to another has not been proved.

Spontaneous development of bacteria does not occur; all bacteria originate from bacteria. There is no other form of development.

The term **bacteria** is used to designate rod-shaped schizomycetes, it is also used when referring to the entire group of schizomycetes, and in its broadest sense includes other higher groups of organisms conveniently classified as "higher forms of bacteria."

The higher bacteria embrace the actinomyces, caldothrices, leptothrices, blastomycetes and hyphomycetes (see page 135).

Bacteria may be differentiated and classified according to their physiology, some species otherwise indistinguishable have peculiar secretions and others are distinguished by the physical and chemical changes they produce in the medium upon which they grow.

For those whose work frequently necessitates the identification of bacteria of soil, water and foodstuffs, "A Manual of Determinative Bacteriology" by F. D. Chester (The Macmillan Co., New York) is a most valuable aid.

It is highly important that as soon as possible we can arrive at the use of a proper and uniform nomenclature for bacteria. At the present time, unfortunately, such is not in use. Advanced students are earnestly recommended to consider this subject and to read especially "Vuillemin—Genera Schizomycetum, Annales Mycologici, xi, 512–527." Also Buchanan, R. E., Journal of Bacteriology, vol. i, No. 6, pp. 591–596, November, 1916.

CHAPTER III

MICROSCOPY

Bacteriology is an outgrowth of microscopy. For many years the study of bacteria was confined to microscopic observations. At present other methods described in the following chapters, are also employed but microscopic studies are still a large and indispensable portion of most investigations.

For ordinary bacteriological work any of the microscopes of standard make do equally well, whether of American or European manufacture. If but one eyepiece or ocular is obtained with the microscope, a 4 or 6 will be found most satisfactory. At least three objectives are required, 2/3 inch, 1/6 inch and 1/12 inch. The 1/6-inch should have a working distance that will permit blood-cell counting and the 1/12 inch objective is an oil immersion lens. Although a mechanical stage is not essential, it is a great convenience and often a time-saving adjunct.

Microscopes are constructed to rest upon and be supported by the base. When it is necessary to move or carry one from place to place the safest way is to grasp the instrument below the stage; never lift it by the barrel, coarse adjustment or fine adjustment. Hold it upright, do not tilt it; failure to observe these precautions in handling, often results in injury to the instrument.

Dust, acid fumes and continuous exposure to direct sunlight are injurious; therefore, when not in use a microscope is protected by a suitable covering.

Proper illumination is very important. Direct sunlight is not desirable, north light, especially from a white cloud, is the best natural light. If gas light is used it should come from a Welsbach burner. Incandescent electric lights should have frosted globes and be 100 candlepower or more.

When unstained preparations are to be examined, as in the observation of hanging-drops, agglutination tests and red blood-cell counting, oblique illumination will generally be most satisfactory; when examining stained bacteria and tissue, central light is best.

ILLUSTRATION OF MICROSCOPE INDICATING THE VARIOUS PARTS

To bring the rays of light upon the object to be examined, the diaphragm must be open. Beginners frequently overlook this. The mirror is manipulated until the best possible illumination is obtained, using the plane side of the mirror usually. Then by focusing the condenser, one determines at what distance from the object it gives best results. Start with the condenser flush with the stage.

When using a low-power objective, the 2/3 or 1/6, one may look in the ocular and focus downward until the field is in focus. This, however, is a dangerous procedure when using a high-power objective.

If the oil immersion lens is to be employed, first place a drop of immersion oil on the slide or cover glass and with the eye fixed on the objective focus downward until the objective touches the oil and almost, but never quite touches the cover glass. Then look in the ocular and slowly focus **upward** until the field comes into view. Never touch the cover glass with the lens.

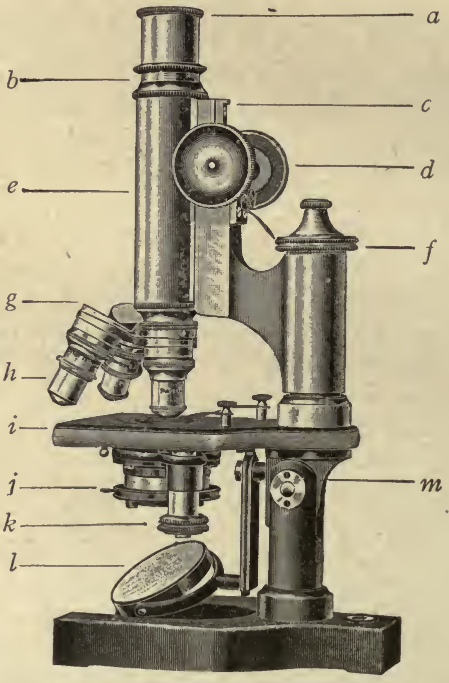

FIG. 1.—MICROSCOPE SUITABLE FOR GENERAL PATHOLOGIC AND BACTERIOLOGIC WORK. *a.* Ocular or eye-piece. *b.* Draw-tube. *c.* Rack. *d.* Milled head of pinion moving the rack; the rack and pinion (*c* and *d*) together are called the coarse adjustment. *e.* Microscopic tube. *f.* Micrometer screw by which the fine adjustment is operated. *g.* Triple nose-piece or revolver which receives the objectives, *h*; in the above instrument there are three objectives which in turn may be rotated into the optical axis. *i.* Stage on the upper surface of which are clips for holding the slide during examination. *j.* Iris diaphragm in substage condenser; the diaphragm permits variation in the quantity of light admitted, and the condenser properly focuses the rays on the object examined. *k.* Screw for raising and lowering the condenser by which the latter, when not in use, may be thrown to the side. *l.* Mirror for reflecting light into the optical axis of the instrument. *m.* Inclination joint permitting inclination of the instrument. The vertical column below the inclination joint is called the pillar and is solidly joined to the large, heavy, horseshoe base supporting the instrument.

A focus is first obtained with the **coarse adjustment;** not until the best focus possible to obtain with it has been reached, do we use the fine adjustment.

The fine adjustment has a very limited range and should never be given more than half a turn one way or the other. It is delicate, easily injured, and once out of order requires the services of a skilled mechanic to adjust it.

When any part of the microscope becomes loose or out of order, unless one is experienced in correcting such faults, it is best to return the instrument to the maker for repairs.

Important as proper illumination and focusing are, satisfactory observations cannot be made if the lenses be dirty, therefore, they are carefully protected from dust and moisture. Keeping an ocular in the instrument when not in use, prevents dust from entering the tube and objective. When the field of vision is obscured by specks, they are usually upon the ocular. To determine this, rotate the ocular while looking through it. If the dust is on the ocular, the specks move when the ocular does. Wipe off the most exposed surface first, if that does not correct the fault, carefully clean all the surfaces of the ocular lenses, one after the other until the ocular is perfectly clear. Should an objective be dirty, first clean the outside, if there is dust inside an ocular, try to remove it with a fine camel's hair brush. Never take an ocular apart; if that seems necessary, let the maker do it. Only fine, soft, clean linen or silk or Japanese lens paper should be used to wipe lenses. They should never be rubbed hard. If a solvent is required to clean lenses, wet the lens paper with xylol or chloroform, nothing else, and rapidly dry the lens after wiping. Immersion oil should never be left on a lens when not in use; it should be wiped off. The substage condenser and mirror require the same careful attention and must be as clean as oculars and objectives. Should a lens be scratched or its finish etched or marred, the services of a lens grinder will be required to restore it.

THE DARK-FIELD MICROSCOPE

Unstained microörganisms are most distinctly visible and their motility and morphology can be most accurately observed when they are situated in a medium that has a *dark background* and are illuminated by rays of light that pass obliquely through the fluid between slide and cover glass without entering the objective. In fact, organisms such as the treponema pallidum, which cannot be satisfactorily observed in ordinary hanging-drop preparations, are distinctly visible and clearly outlined in dark-field preparations, notwithstanding the fact that magnification is the same in each case.

For this desirable method of observation one requires a microscope fitted with a dark-field condenser. Such a condenser can be affixed to any ordinary microscope but on account of the time necessary to properly adjust the microscope for this work, the only practical method for routine examinations, at present, is to equip a microscope with a dark field, adjust it, and fix it in position and only use it for this method of observation.

The one great value of this apparatus, at present, in routine diagnostic work, is in the examination of fluids and scrapings from suspicious lesions to detect the presence of the treponema pallidum.

More intense illumination is required for the dark-field microscope than for the ordinary and can be obtained most satisfactorily from one of the numerous electric lamps made especially for the purpose. A lens or round flask filled with water must be so placed between the source of light and the microscope, that by manipulating the mirror of the microscope it is possible to get the whole surface of it brightly and uniformly illuminated, at the same time the image of the source of light appears clearly defined on the mirror.

The dark field must be accurately centered by moving the screws on the side of it, while looking through a low-power objective (without eyepiece in tube) until the center of the field is brightly illuminated and all shadows and haloes disappear.

When proper adjustment has been achieved, the exact location of lamp, condenser and microscope on the table should be noted.

Though not absolutely necessary, it is much more satisfactory to make observations with this microscope in a dark room than elsewhere.

Only slides and cover glasses of the thickness for which the apparatus has been constructed should be used.

PREPARATION OF SPECIMENS FOR EXAMINATION

In producing fluid or scrapings for examination for treponema pallidum, it is important to avoid bleeding and to obtain the specimen free of blood. Fluid can be obtained from macules and papules by gently pinching them with a hemostat and nicking the elevated surface with a sharp knife. If done carefully, several drops of clear serum, free of blood cells, can be expressed. Specimens can similarly be obtained from indurated surfaces and the margins of ulcers.

Lesions should not be washed or dressed with any antiseptic for at least 24 hours before specimens are obtained. About 6 hours before obtaining the fluid they should be gently cleansed with normal salt solution and covered with a gauze dressing, moistened with salt solution, which dressing is not disturbed until the material for examination is collected.

From macules, papules and ulcers, material for examination can be collected and handled most easily and safely with straight, capillary glass tubes, about 6 inches long (Wright's tubes). Fluid from enlarged glands is withdrawn with a syringe and needle.

Examination of such specimens should be made as soon as possible—never more than an hour—after they are collected.

A drop of warm, sterile, salt solution is placed on the center of each of a number of perfectly clean slides, and a drop of the fluid or scrapings to be examined is mixed with it. The mixture should form a thin film, free of air bubbles, and occupy the entire space between the slide and the cover glass that is dropped upon it.

The preparations are luted with melted vaseline or paraffin, a drop of immersion oil is placed on the dark-field condenser and the slide placed upon it, another drop of immersion oil is placed on the cover glass and the oil immersion lens brought into contact with it. The preparation is now ready to examine when brought into focus.

SLIDES AND COVER GLASSES

Slides and cover glasses when purchased are seldom, if ever, clean enough for use. The easiest and most satisfactory method of cleaning is to immerse in spiritus ætheris nitrosi, dry and polish with fine linen or paper. No matter how packed, slides and covers cleaned in bulk for future work always collect some dirt before they are required. To avoid unnecessary repetition, slides and cover glasses are placed in jars containing spiritus ætheris nitrosi and dried and polished immediately before use.

When bacteria are to be examined unstained and particularly when motility or agglutination is to be observed, the best method is to make a **hanging-drop** preparation. For this purpose slides are made with a concave depression in the center of one side. A drop of the fluid to be examined is placed on the center of a cover glass, the cover glass is inverted and placed over the cavity of the slide so that the drop of fluid hanging on it is suspended in the depression on the slide, without touching the slide at any point. To prevent the slide from moving and to exclude air several tiny drops of immersion oil or vaselin are placed on the slide near the concave depression, before placing the cover glass upon it. It is important that the drop of fluid be on the center of the cover glass, suspended in the concave depression without touching the slide, and that the slide be kept level, neither tilting it before placing on the stage of the microscope nor while it is there. Oblique and not too bright illumination is best for the study of hanging-drops.

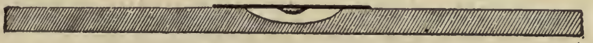

FIG. 2.—HANGING-DROP PREPARATION.

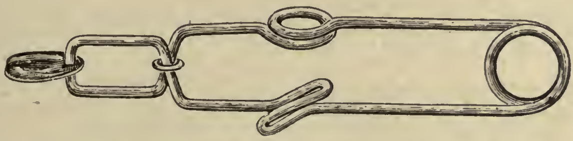

FIG. 3.—STEWART'S COVER-GLASS FORCEPS

Method of holding cover-glass and applying the stain. Convenient for blood work, and absolutely necessary for bacteriologic work; useful also in sputum examination, etc. The lower instrument shows a cover-glass with stain in position. If the cover-glass be grasped with the film or spread side toward the side of the forceps that has the circular bend intended to fit the thumb, shown at A, there will be no danger of losing trace of the side containing the spread, as this side of the forceps is practically always upward.

Bacteria are most commonly examined after staining and must first be fixed to the slide or cover glass to prevent subsequent washing from carrying them away.

If it is fluid one wishes to examine, as when the bacteria are in bouillon, milk, etc., a small drop is placed on a perfectly clean cover glass (which has been flamed and allowed to cool) and spread in a thin even film with a platinum loop. Sputum, pus and similar viscid substances are more readily spread in a thin even film by placing a drop on the center of a slide or cover glass then dropping a second slide or cover glass on top of it and allowing the material to spread out between them, then slide the glasses apart.

When dried substances and bacteria removed from solid culture media are to be examined, a drop of sterile distilled water is placed on a slide or cover glass, the dried matter or bacteria picked up with a sterile platinum loop and the loop lightly touched to the drop of water until a slight cloudiness appears. The excess is then burned off the loop and when it is cool, the bacteria are mixed with the drop of water and it is spread out in a thin film with the loop.

When the matter to be examined has been spread on the slide or cover glass, whether fluid as bouillon, viscid as sputum, or solid mixed with water, it is fixed by drying. This is commonly done by gently heating over a flame; should the slides be placed nearer the flame than one can hold one's hand with comfort, the organisms may be destroyed or so altered as to become invisible. Some experienced observers state that of two sets of slides smeared from one sample of sputum, one set dried or fixed by gently heating over a flame, the other set fixed by simply permitting them to stand at room temperature until dry, often those fixed without heating will show tubercle bacilli when those fixed by flame do not. Some workers prefer fixing their preparations by placing them in a thermostat until dry.

Whether smears shall be made upon cover glasses or slides is a matter of personal choice, determined by convenience. Slides are less fragile and easier to handle than cover glasses while making the smears; on the other hand, cover glasses are easier to handle when staining.

If smears are made on slides, when fixed, stained and dried they can be examined without mounting a cover glass; cover glasses when ready for examination must, of course, be mounted on slides.

The essential points in making smears for microscopic examination are as follows:

1. Use absolutely clean slides and covers.
2. Spread the material in a thin, even, transparent film.
3. Protect it from contamination with dust, etc.
4. Guard against overheating during fixation.

Cover glasses are usually attached to slides with Canada balsam which should be free of acid. A drop of balsam is placed on the slide and the cover glass placed upon it. The balsam should be free from air bubbles and dirt. If it has the proper consistency it will spread out in a thin film. There should be no appreciable elevation of the cover above the slide. When balsam becomes so thick that it will not spread unless pressure is used, it should be thinned out by adding to it xylol.

Slides can be used repeatedly, if thoroughly cleaned. A satisfactory method is as follows:

Keep a jar containing a piece of toilet soap and water on the microscope table; when through with slides, place them in jar. After 48 hours wipe them clean and place in running water for ¼ hour, wipe dry and store in a jar containing spiritus ætheris nitrosi.

After a time slides that have been used and cleaned repeatedly become scratched; scratched slides should never be used when differential staining is to be done.

In a general laboratory new slides should be used for bacteriological work; afterward they should be set aside for tissue and other work.

CHAPTER IV

STAINING

Organisms so small that they are almost or entirely invisible before staining, become distinctly visible when stained. Details unnoticeable in unstained bacteria are apparent in the stained. By staining we are enabled to distinguish some species from others because of differences in their ability to absorb and retain certain stains, and to resist the decolorizing action of acids and alcohol.

Aqueous solutions of anilin dyes are most commonly employed in staining bacteria. Stains and methods of staining are numerous. Fortunately, however, there are about a half dozen stains and methods of staining which suffice for all ordinary bacteriological examinations.

The term cubic centimeter (cc.) as used in this volume is synonymous with the term mil. The United States Pharmacopeia IX has brought about a change from the former to the latter, this being an abbreviation for the term milliliter (the first three letters of which are mil). The United States Bureau of Standards regards the term cc. as a misnomer, there being a slight difference between the thousandth part of a liter and the cc. In addition the change was brought about to promote international conformity.

As this volume will be used by students and practitioners familiar with the term cc., and not so familiar with the better term, mil., it has been thought advisable to still retain cc. in this edition.

LOEFFLER'S METHYLENE BLUE

1:10,000 (1/100 per cent.) aqueous sol. of potassium hydrate, 10 cc.

Saturated alcoholic solution of methylene blue, 3 cc.

Loeffler's methylene blue is a very satisfactory stain for most bacteria. The method of staining is as follows:

1. Cover the preparation to be stained with Loeffler's methylene blue, allow the stain to remain on the specimen for 1 to 5 minutes.
2. Wash the stain off with water, and allow the slide or cover glass to dry.
3. Mount.
4. Examine specimen.

FUCHSIN

Saturated alcoholic solution of basic fuchsin, 5 cc.

Distilled water, 95 cc.

Apply this stain in the same manner as Loeffler's methylene blue.

GENTIAN VIOLET

Saturated alcoholic solution of gentian violet, 5 cc.
Distilled water, 95 cc.
Apply the same as Loeffler's methylene blue.

EOSIN

Eosin, 1/10 to 1/4 Gm.
Distilled water, 100 cc.
Allow stain to remain on the specimen for 1 minute, then wash off with water.

Gram's method of staining permits the differentiation of organisms otherwise indistinguishable under the microscope, and is, therefore, of great service.

GRAM'S METHOD

1. Stain specimen with anilin gentian violet for 3 to 5 minutes.
2. Wash with water to remove excess stain.
3. Apply Gram's solution for 2 minutes.
4. Wash specimen with 95 per cent. alcohol until specimen gives off no more color.
5. Dry and examine.

Gram positive bacteria (Gram +) remain a deep violet, almost black.

Gram negative bacteria (Gram −) are colorless.

When the anilin gentian violet is applied to a slide or cover glass, all the bacteria on it are stained violet. When the alcohol is applied it does not affect the Gram positive bacteria, they remain violet, but the alcohol takes the violet stain out of the Gram negative bacteria, or in other words, decolorizes them.

As unstained bacteria are almost indiscernible, a counter stain is usually applied after staining by Gram's method.

	GRAM POSITIVE	GRAM NEGATIVE
	COCCI	
Micrococci of Suppuration	Staphylococcus pyogenes albus	
	Staphylococcus pyogenes aureus	
	Staphylococcus pyogenes citreus	
	Micrococcus pyogenes fœtidus	Gonococcus
	Micrococcus cereus albus	Meningococcus
	Micrococcus cereus flavus	Micrococcus catarrhalis
	Micrococcus epidermidis albus	Micrococcus melitensis
	Streptococci	
	Pneumococci	
	Sarcina lutea	
	Sarcina alba	
	Sarcina aurantiaca	
	Micrococcus tetragenus	

	GRAM POSITIVE	GRAM NEGATIVE
BACILLI (non-spore-bearing)	Corynebacteria Diphtheria bacillus Pseudodiphth. bacilli Xerosis bacillus Tubercle bacillus Bacillus lepræ	Bacillus of Friedlander Bacillus of influenza Bacillus of pertussis (Bordet-Gengou) Bacillus of Morax and Axenfeld Kock-Weeks bacillus Bacillus pestis. Typhoid bacillus (Eberth) Paratyphoid bacilli Bacillus coli Bacillus icteroides Bacillus of dysentery (Shiga's) and allied organisms Bacillus proteus vulgaris and other members of the proteus group Bacillus pyocyaneus Bacillus prodigiosus Bacillus mallei (glanders) Bacillus of soft chancre (Ducrey's)
BACILLI (spore-bearing)	Bacillus of tetanus Bacillus aerogenes capsulatus Bacillus anthracis Bacillus botulinus Bacillus subtilis	Bacillus of malignant edema Bacillus of symptomatic anthrax
SPIRILLA		Spirillum and bacillus of Vincent Spirillum of Asiatic cholera Spirillum and allied organisms Spirochæta of Obermeier Treponema pertenuis Treponema pallidum
HIGHER BACTERIA	Leptothrices Cladothrices Streptothrices Saccharomycetes (blastomycetes) Hyphomycetes (mucorini)	

ANILIN WATER

Anilin water is prepared by adding anilin oil to distilled water, shaking after the addition of each drop. The oil is added until the water is saturated. This water must be filtered until entirely free from visible globules of oil, even though several passages through filter-paper be necessary.

ANILIN GENTIAN VIOLET

Anilin water, 84 c.c.

Saturated alcoholic solution of gentian violet, 16 cc.

Anilin gentian violet rapidly deteriorates and in a few days or weeks is useless.

GRAM'S SOLUTION

Iodine, 1 Gm.

Potassium iodide, 2 Gm.

Distilled water, 300 cc.

In routine work Loeffler's methylene blue is generally used and found most satisfactory for staining the diphtheria bacillus, but one of the characteristics of this organism, irregular staining, is best brought out by Neisser's method of staining.

When stained with Loeffler's methylene blue, one, two or more portions of the diphtheria bacillus stain deeply, the rest of the organism stains faintly or not at all, thus giving a deceptive impression of its contour. With the two extremities stained deeply and the intermediate portion stained faintly or not at all, so-called polar staining, or with both poles and the center stained deeply and the portions between the center and poles unstained, so-called barred staining or beaded forms, the organism may appear more like several cocci in a row than a bacillus, which it really is.

By Neisser's method the entire bacillus is stained, the ends or the ends and center blue, intermediate portions light brown. Two solutions are used to obtain this effect, as follows:

NEISSER'S METHOD

1. Apply Neisser's acid methylene blue to specimen for 2 to 5 minutes.
2. Wash with water to remove excess stain.
3. Apply Bismarck brown for 1 minute.
4. Wash with water.
5. Dry, mount and examine.

NEISSER'S ACID METHYLENE BLUE

1. Methylene blue, 0.1 Gm.
2. Alcohol, 2 cc.
3. Glacial acetic acid, 5 cc.
4. Distilled water, 95 cc.

First mix the methylene blue and alcohol together, then mix the glacial acetic acid and distilled water together, finally add the acid solution to the alcoholic solution, and filter.

BISMARCK BROWN

Bismarck brown, 0.2 Gm.

Boiling distilled water, 100 cc.

Mix together and filter.

Most bacteria when stained can be decolorized by washing with acids. But there are some bacteria that cannot be decolorized by acids after they have been stained. These we call **acid-fast.** A number of organisms which are acid-fast can be decolorized with alcohol. They are acid-fast, but not alcohol-fast. Of the pathogenic organisms there are but two which are both acid-fast and alcohol-fast, the tubercle bacillus and the leprosy bacillus.

It has been recently stated that there are non-acid fast and non-alcohol-fast strains of the leprosy bacillus. Practically, the tubercle bacillus is always acid- and alcohol-fast; but the degree of resistance to acid and alcohol of some other organisms at times simulates that of the tubercle bacillus, so that rare occasions arise when differentiation can only be determined by cultural test.

To demonstrate acid-fast bacilli, apply carbol fuchsin for 15 minutes, wash with 25 per cent. aqueous solution sulphuric acid until specimen gives off no more color, wash with water, apply Loeffler's methylene blue for 3 to 5 minutes, wash in water, dry and examine.

When the carbol fuchsin is applied everything is stained red. The acid removes the stain from all but the acid-fast organisms, these remain red, the others are decolorized. When the methylene blue is added it does not affect the acid-fast organisms, they are already saturated with fuchsin and remain red, but it tints everything else blue, so that the acid-fast organisms appear red and the non-acid-fast organisms blue.

Bacteria that are decolorized by acids are also decolorized by alcohol.

To distinguish bacteria that are acid-fast but not alcohol-fast, from those which are both acid- and alcohol-fast, make several slides, stain some with carbol fuchsin 5 to 15 minutes, apply 25 per cent. aqueous solution of sulphuric acid for 2 or 3 minutes, wash with water and apply Loeffler's methylene blue. Acid-fast organisms will appear red. The remaining slides stain with carbol fuchsin as before, then apply either absolute alcohol or 5 per cent. alcoholic solution of sulphuric acid or 3 per cent. alcoholic solution of nitric acid for 10 to 15 minutes. Wash with water and counter stain with Loeffler's methylene blue. Bacteria that are both acid-fast and alcohol-fast will appear red, those which are acid-fast but not alcohol-fast will appear blue.

In diagnostic work, when examining sputum, etc., for tubercle bacilli, time and labor are commonly saved by combining counter stain and decolorizing agent, as in Gabbet's solution and Pappenheim's solution, which are the ones commonly used. After staining with carbol fuchsin 5 to 15 minutes, Pappenheim's solution is applied for 15 minutes, then wash in water, dry and mount. Organisms which are both acid- and alcohol-fast, appear red, the others blue.

When Gabbet's solution is used it is applied for 2 minutes, after staining with carbol fuchsin. The specimen is then washed with water, dried and examined. Those organisms which are only acid-fast, as well as those which are acid-fast and alcohol-fast, appear red, other bacteria blue.

CARBOL FUCHSIN

Saturated alcoholic solution of fuchsin, 1 cc.
Five per cent. aqueous solution of carbolic acid, 9 cc.

PAPPENHEIM'S SOLUTION

Coralin (rosalic acid), 1 Gm.
Absolute alcohol, 100 cc.

Mix acid and alcohol together; add methylene blue to saturation and 20 cc. of glycerin.

GABBET'S SOLUTION

Methylene blue, 2 Gm.
25 per cent. aqueous solution sulphuric acid, 100 cc.

The flagella possessed by motile bacilli cannot be demonstrated by any of the methods of staining previously described. Many stains have been devised especially for this purpose; McCrorie's is probably the best.

McCRORIE'S FLAGELLA STAIN

Saturated alcoholic solution of night blue, 1 cc.
10 per cent. aqueous solution of tannic acid, 1 cc.
10 per cent. aqueous solution of alum, 1 cc.

Eighteen-hour-old agar cultures are used. Thin spreads are carefully dried at a temperature not greater than 50°C. Apply stain for 2 minutes, then gradually heat until steam rises, wash, dry and examine.

The demonstration of flagella is difficult and of no practical importance.

To stain the capsule with which some organisms are surrounded requires special stains and Muir's method will be found satisfactory. Only very thin films should be made to obtain capsule staining.

MUIR'S METHOD

1. Stain with carbol fuchsin for 30 seconds, and steam.
2. Wash with 95 per cent. alcohol for several seconds.
3. Wash with water.
4. Apply mordant for 5 to 10 seconds.
5. Wash in water.
6. Wash with alcohol for 1 minute.
7. Wash in water.
8. Counter stain with methylene blue for ½ minute.
9. Dehydrate in alcohol.
10. Clear in xylol and mount.

MORDANT FOR CAPSULE STAINING (MUIR'S METHOD)

Saturated aqueous solution mercuric chloride, 2 cc.

25 per cent. aqueous solution tannic acid, 2 cc.

Saturated aqueous solution potash alum, 5 cc.

Spores do not stain by the methods generally employed for bacteria.

When a bacillus containing an endospore is stained with eosin, fuchsin or methylene blue, the bacillus red or blue, the spore colorless, by contrast it is apparent.

To stain the spores and maintain the contrast, use the following method:

METHOD FOR STAINING SPORES

1. Stain with carbol fuchsin for 15 minutes.
2. Wash with 1 per cent. aqueous solution of sulphuric acid until bleaching is noticed.
3. Wash in water.
4. Apply methylene blue for 30 seconds.
5. Wash in water, dry and examine.

Spores will be red and bacteria blue.

BLOOD-CELL STAINING

It is occasionally necessary to stain blood, serous fluids or exudates containing bacteria by a method that will show and differentiate the white blood cells. For this purpose the preparations are made and stained by the methods commonly used in the examination of blood cells:

A drop of the blood or serous fluid is placed on the center of a square cover glass and a second cover glass dropped diagonally on it. As soon as the fluid ceases to spread the glasses are slid apart; or, a drop of the fluid is placed on a slide, near one end, and is drawn along the slide with the edge of a second slide held at an angle of about 45°. By whichever method the spread is made, it must be done rapidly and the film must be thin and even. It is gently heated until dry and then stained with Romanowsky's, Giemsa's, or any of the modifications of these. The preparation is covered with the stain, from 1 to 2 minutes later distilled water is added to the stain until a metallic scum appears, usually it requires as much water as stain to effect this: 5 to 10 minutes later the slide or cover glass is washed with water, dried and examined.

Saturated alcoholic solutions of fuchsin, methylene blue and gentian violet keep until used. It is a great convenience to have such solutions in stock. They should be stored in dark glass-stoppered bottles. Absolute alcohol is used in their preparation and the addition of 10 Gm. of powdered stain to each 100 cc. of alcohol is more than sufficient to saturate it. When made saturated, solutions are thoroughly shaken and then allowed to sediment for at least 24 hours before use. Care must afterwards be taken when pouring that no undissolved particles get into staining fluids. These latter must all be perfectly clear and free of undissolved particles. When procuring stains it is best to specify "Grubler's."

GIEMSA'S STAIN

Azur II-eosin	3.0 Gm.
Azur II	0.8 Gm.
Glycerin (C.P.)	250.0 cc.
Methyl alcohol (C.P.)	250.0 cc.

Grind up dyes in alcohol, then add glycerin. Fix film in methyl alcohol, stain 5 minutes with:

Giemsa	14 gtt.
H_2O	10 gtt

For the principles and mechanism of staining of microörganisms, consult "The Microtomist's Vade-Mecum," A. B. Lee, P. Blakiston's Son & Co., Philadelphia.

CHAPTER V

STERILIZATION

Sterilization is the removal of germs from matter or destruction of germs upon or in matter. It can be accomplished in the case of fluids, by filtration, the addition of germicidal chemicals or by heat. Solids can be sterilized by the addition of germicidal chemicals or by heat.

Filtration can sterilize under the following conditions:

1. When the pores of the filter are too small to permit the passage of bacteria.
2. When the filtrate, uncontaminated, flows into a sterile container.
3. When the construction of the filter and the connection of filter to filtrate container are such as to prevent unfiltered fluid and air from mingling with the filtrate.
4. When the filtrate is sealed in its container before exposure to contamination with germs.

To secure sterilization by this method requires the employment of a properly selected, tightly adjusted and nicely manipulated filter and filtrate container. Not easy for a tyro to do.

In routine laboratory work, this method is seldom employed except in the sterilization of therapeutic sera and such solutions as would be injured by heat. The filters used are unglazed porcelain tubes which are first tested by passing through them, fluids known to contain bacteria and then testing the filtrate for sterility.

Chemical Sterilization commonly referred to as disinfection, is accomplished by submerging utensils in a germicidal solution, or by mixing germicidal solutions with fluid and solid substances.

The efficacy of this method depends upon the following:

1. The selection of a chemical which can kill germs.
2. The selection of a chemical which is not changed to an inert substance, without germicidal power, when brought into contact with the matter contaminated with germs.
3. The use of sufficient quantity of germicide to destroy all germs.
4. Direct contact of germicidal agent with every germ.
5. Maintenance of the contact for a sufficient length of time.
6. The rate at which chemical sterilization progresses is in proportion to the hydrogen ion concentration, and to some extent, increased by the presence of sodium chloride.

Chemical sterilization is restricted by these conditions which must be fulfilled. It is further restricted by the uncertainty of results. Its employment is limited because most germicidal chemicals cannot be easily removed from substances with which they have been mixed, therefore, although sterilized, the substance still remains unfit, if intended for human consumption, therapeutic administration, or for use as culture medium, upon which to grow bacteria.

Chemical sterilization is principally employed in the treatment of refuse matter which may contain pathogenic organisms. Chloroform and ether, which can be removed by a slight degree of heating, are occasionally used for chemical sterilization, as in the sterilization of serum.

The rate at which chemical sterilization progresses is in proportion to the hydrogen ion concentration. This consideration will explain the greater activity shown by chemicals in aqueous solutions than in alcoholic or ethereal admixture, since it has been shown that ionization is more pronounced and greater in water than in other solutions. Sodium chloride and other chemical salts when added to the solution of an inorganic disinfectant seem to retard the action of the latter, inasmuch as these salts tend to decrease the concentration of free ions. However, their presence with the organic disinfectants, notably the phenols and coal-tar derivatives, tends to increase the bactericidal powers of such preparations. This fact is sufficient proof that the phenols and their allied members act as an entire molecule and not as individual ions.

DISINFECTANTS USED IN SOLUTION

How disinfectants effect the injury of bacteria is a problem still in doubt. Some seem to act by their readiness in coagulating the cell protoplasm. Others act by their power of rapidly oxidizing the bacterial body. Some may produce injury to the cell by their property of withdrawing water from its tissues, while still others exert their toxic effect by penetrating the cell wall and inactivating the cell protoplasm.

ACIDS AND ALKALIES

The strong inorganic acids and alkalies are active germicidal agents, due to the fact that they readily ionize. The acids possess this property to a greater extent than the latter.

Bichloride of mercury is one of the most extensively used antiseptics. Although highly efficient under certain conditions, it is so readily destroyed and its efficiency lowered as commonly used that it becomes an undependable disinfectant. It is poisonous, irritating and highly toxic. It is precipitated by soap and its precipitating properties by albumen limits its use as a penetrating agent.

Peroxide of hydrogen has been used extensively, because of its oxidizing properties. Regarded as very efficient by some, it is considered worthless by others. This is because of its rapid destruction in the presence of organic matter (especially blood, pus, etc.), so that no great dependence can be placed upon its disinfectant properties, unless used in excessive amounts.

Permanganate and dichromate of potash are two inorganic salts, which as oxidizing agents, are powerful disinfectants. These are also rapidly reduced and inactivated, as far as their bactericidal properties are concerned, by organic matter.

Silver salts.—The silver salts, as silver nitrate, argyrol and protargyrol, are of a special value as disinfectants for some of the cocci. This fact explains the extensive use of these chemicals in the treatment of gonorrheal ophthalmia and certain types of sore throat.

The Halogens.—Perhaps more commonly used than any of the foregoing inorganic disinfectants are the free halogens, notably iodine and chlorine, and to a minor extent free bromine.

Iodine, both as terchloride of iodine (ICl_3) and free iodine are extremely strong disinfectants. Tincture iodine (U.S.P.) or 10 per cent. is a simple and most efficient method of sterilizing the skin.

Chlorine, both free and available, is perhaps one of the most common of inorganic germicides.

Chloride of lime, or the so-called "bleaching powder," is a substance, possessing probably the formula $CaOCl_2$. The action of dilute acids or air (in which we find CO_2 and moisture, thus forming carbonic acid) upon this substance results in the liberation of chlorine, so termed "available." The latter, because of its readiness in destroying vegetative forms of bacteria, when even in dilute solutions, has brought about the extensive use of this chemical in the disinfection of sewage and water supplies.

A preparation which has been known for years, but recently exploited and used more extensively, is the *Dakin-Carrel solution.* This product is made like Labarraque's solution of the United States Pharmacopeia, but possessing special precautionary differences, as may be observed below, where follow its details and mode of preparation. As this solution liberates its chlorine when in contact with wounds and retains its antiseptic properties for a long time, it will be valuable only when it is in no way harmful to the cells of the surrounding tissues. This can be taken care of by observing that the concentration of available chlorine be uniform and of the standardized strength, and that the product be free from irritating substances, as free alkali, boric acid, etc. This preparation, when used in surgery, is employed by immersing all the wound surfaces through a constant flow, maintained by a hydraulic system. At the same time, all portions of the skin surrounding the wound are protected from exposure to the chlorin solution, by having applied to it sterile gauze strips with vaseline.

PREPARATION OF DAKIN'S SOLUTION (DAUFRESNE'S TECHNIQUE)

Original letter by Dr. Carrel

(Journal of the American Medical Association, Dec. 9, 1916, p. 1777)

"Dakin's solution is a solution of sodium hypochlorite for surgical use, the characteristics of which, established after numerous tests and a long practical experience, are as follows:

(*a*) **"Complete Absence of Caustic Alkali.**—The absolute necessity for employing in the treatment of wounds a solution free from alkali hydroxide excludes the commercial Javelle water, Labarraque's solution and all the solutions prepared by any other procedure than the following:

(*b*) **"Concentration.**—The concentration of sodium hypochlorite must be exactly between 0.45 and 0.50 per cent. Below 0.45 per cent. of hypochlorite the solution is not sufficiently active; above 0.50 per cent. it becomes irritating.

"Chemicals Required for the Preparation.—Three chemical substances are indispensable to Dakin's solution: Chlorinated lime, anhydrous sodium carbonate and sodium bicarbonate. Among these three products the latter two are of a practically adequate constancy, but this is not the case with the first. Its content in active chlorine (decoloring chlorine) varies within wide limits, and it is absolutely indispensable to titrate it before using it.

"Titration of the Chlorinated Lime.—There must be on hand for this special purpose:

A 25-cc. buret graduated in 0.1 cc.

1 pipette gaged for 10 cc.

A decinormal solution of sodium thiosulphate (hyposulphite). This decinormal solution of sodium thiosulphate can be obtained in the market; it can also be prepared by dissolving 25 Gm. of pure crystalline sodium thiosulphate in 1 liter of distilled water, and verifying by the decoloration of an equal volume of the decinormal solution of iodine by this solution. The iodine is prepared by dissolving 1.27 Gm. iodine and 5 Gm. potassium iodide in 100 cc. of water.

"The material for the dosage thus provided, a sample of the provision of chlorinated lime on hand is taken up either with a special sound or in small quantities from the mass, which then are carefully mixed.

"Weigh out 20 Gm. of this average sample, mix it as completely as possible with 1 liter of ordinary water, and leave it in contact for a *few* hours, agitating it from time to time. Filter.

"Measure exactly with the gaged pipette 10 cc. of the clear fluid; add to it 20 cc. of a 1 : 10 solution of potassium iodide and 2 cc. of acetic or hydrochloric acid. Drop, a drop at a time, into this mixture a decinormal solution of sodium thiosulphate until decoloration is complete.

"The number of cubic centimeters of the hypochlorite solution required for complete decoloration, multiplied by 1.775, gives the weight of the active chlorine contained in 100 Gm. of the chlorinated lime.

"This figure being known, it is applied to the accompanying table, which will give the quantities of chlorinated lime, of sodium carbonate and of sodium bicarbonate which are to be employed to prepare 10 liters of Dakin's solution.

"Preparation of Dakin's Solution.—To prepare 10 liters of the solution:

1. "Weigh exactly the quantities of chlorinated lime, sodium carbonate and sodium bicarbonate which have been determined in the course of the preceding trial.

2. "Place in a 12-liter jar the chlorinated lime and 5 liters of ordinary water, agitate vigorously for a few minutes, and leave in contact for from 6 to 12 hours, over night, for instance.

QUANTITIES OF INGREDIENTS FOR TEN LITERS OF DAKIN'S SOLUTION

Titer of chlorinated lime	Chlorinated lime, Gm.	Anhydrous sodium carbonate, Gm.	Sodium bicarbonate, Gm.
20	230	115	96
21	220	110	92
22	210	105	88
23	200	100	84
24	192	96	80
25	184	92	76
26	177	89	72
27	170	85	70
28	164	82	68
29	159	80	66
30	154	77	64
31	148	74	62
32	144	72	60
33	140	70	59
34	135	68	57
35	132	66	55
36	128	64	53
37	124	62	52

3. "At the same time dissolve, cold, in the 5 other liters of water the sodium carbonate and the bicarbonate.

4. "Pour all at once the solution of the sodium salts into the jar containing the maceration of chlorinated lime, agitate vigorously for a few moments, and leave it quiet to permit the calcium carbonate to settle as it forms. At the end of ½ hour siphon the liquid, and filter it through double paper to obtain an entirely limpid product, which must be protected from light.

"Light, in fact, alters quite rapidly solutions of hypochlorite, and it is indispensable to protect from its action the solutions which are to be preserved. The best way to realize these conditions is to keep the finished fluid in large wicker covered demijohns of black glass.

"**Titration of Dakin's Solution.**—It is a wise precaution to verify, from time to time, the titer of the solution. This titration utilizes the same material and the same chemical substances as are used to determine the active chlorine in the chlorinated lime.

"Measure out 10 cc. of the solution, add 2 cc. of 1:10 solution of potassium iodide, and 2 cc. of acetic or hydrochloric acid. Drop, a drop at a time, into this mixture a decinormal solution of sodium thiosulphate until decoloration is complete.

"The number of cubic centimeters employed multiplied by 0.03725 will give the weight of the sodium hypochlorite contained in 100 cc. of the solution.

"**The Test for the Alkalinity of Dakin's Solution.**—It is easy to differentiate the solution obtained by this procedure from the commercial hypochlorites and from Labarraque's solution.

"Pour into a glass about 20 cc. of the fluid, and drop on the surface a few centigrams of phenolphthalein in powdered form. Dakin's solution, correctly prepared, gives absolutely no change in tint, while in the same conditions Javelle water and Labarraque's fluid give an intense red coloration which indicates in the latter two solutions the presence of free caustic sodium."

Numerous preparations have been put forth to replace the Dakin-Carrel solution. None of them has as yet stood the test, except the one recommended by Duret. His preparation depends upon the liberation of chlorine from magnesium hypochlorite, instead of a sodium salt. This solution is more stable, isotonic with blood serum, and as it has no alkali entering into its preparation, the latter will be free from such contamination. The following is its formula as published in the Journal of the American Pharmaceutical Association (page 241, March, 1917).

Chlorinated lime	28.00 Gm.
Magnesium sulphate	18.20 Gm.
Water	1000.00 Gm.

The two salts are triturated in a mortar and the water added gradually in small portions. The solution is then filtered through cotton-wool.

Solution of eusol, and chloramine, the coal-tar derivative containing available chlorine, have been recommended recently by Dr. Dakin as valuable disinfectants.

A complete report of the use of chloramine in the treatment of infected wounds may be found in the Journal of the A. M. A. July 7, 1917, page 27.

The organic disinfectants are by far more numerous and indeed countless.

Ethyl alcohol is one of the most common disinfectants of this class. By many it is regarded of little value, but experiments recently carried out verify the results of Harrington and Walker (Boston Medical and Surgical Journal, vol. 148, p. 548). These men found that "unless the bacterial envelope contains a certain amount of moisture, it is impervious to strong alcohol, but dried bacteria in contact with alcohol, containing from 30 to 60 per cent. of water, will absorb the necessary amount of water therefrom very quickly and then the alcohol itself can reach the cell protoplasm and destroy it." Adding small portions of chloroform, ether and more commonly acetone (these acting as fat solvent) is commonly practised.

Coal tar has furnished many substances that possess disinfectant properties. Much doubt still prevails as to the efficiency of numerous members of this class on the basis of value assigned them due to laboratory tests, especially since many of them have proven of little value in practice. Though this be a fact, still there remains a large number which are available and possess a high disinfectant value.

Phenol, the most commonly used organic disinfectant, is commonly employed as a wash in strengths from 1 to 5 per cent.

Cresol, ortho-, meta and para, the mixture of the three gives rise to the commonly used **tricresol.** These are more powerfully germicidal than phenol.

Such antiseptic value, freedom from irritation and a low toxicity tend to make it valuable. **Lysol** and **liquor cresolis compositus** (U.S.P.) are preparations of this chemical in a neutral soap, which when dissolved in water form a clear or slightly opalescent liquid. **Creolin** is a similar preparation with a resin soap, forming, however, a turbid emulsion with water.

There are numerous other coal-tar disinfectants, which are more germicidal than phenol. They are valuable deodorants by destroying the putrefactive bacteria producing such odors. They are less toxic, not readily affected by organic matter and much cheaper if we compare their relative cost and disinfectant coefficient with that of phenol.

Formaldehyde is used principally as a gaseous disinfectant. A solution of the gas in water (liquor formaldehydi, U.S.P.) when used as a liquid disinfectant is inferior to phenol or any of the coal-tar products.

Iodoform in itself is a very weak antiseptic, but when applied to wounds, the organic matter present effects the liberation of iodine, which results in bactericidal action.

When certain dyes were discovered to possess a specific staining effect for bacteria, numerous attempts were made to use them as germicidal or antiseptic agents. Drigalski and Conradi observed the inhibitory effect that crystal violet possessed upon the growth of the cocci, while this is also considered when introducing gentian violet in the preparation of Petroff's media. The antiseptic value displayed by brilliant green, crystal violet, and malachite green can be observed in the chart on the following page, as published by Browning, Henaway, Gulbrausen and Thornton. Experiments thus far performed led many to believe that the dyes possess a very low toxicity and at the same time a high antiseptic value.

Flavine, a substance belonging to the latter class, has recently been extensively exploited by the above four co-workers, at the Bland-Sutton Institute of the Middlesex Hospital in England, their original work having been published in the British Medical Journal (No. 2925, Jan. 20, 1917). Flavine is a substance belonging to the acridine group, being chemically the chloride of diamino-methyl acridinum. It is a stable chemical, the solutions of which are not altered at boiling temperature.

"Flavine has been found to possess extremely powerful bactericidal and antiseptic properties, which are enhanced rather than diminished by admixture with serum. In this respect, flavine differs from all other antiseptics in common use.

2. In the presence of serum, flavine is the most potent bactericide of all those investigated for both staphylococcus and B. coli and it is easily efficient for the enterococcus and for anaerobes, such as B. œdematis maligni.

3. Flavine, in its relation to its bactericidal power, is very much less detrimental to the process of phagocytosis and less harmful to the tissues than the other substances; hence much higher effective concentrations can be employed without damaging the tissues or interfering with the natural defensive mechanisms.

4. Clinical results have substantiated the estimate of the therapeutic value of flavine."

The following chart shows the antiseptic value of flavine and other substances, as published by the same experimenters.

GASEOUS DISINFECTION

The best and most ideal way of disinfecting and destroying those agents that produce disease is by the use of a suitable gaseous disinfectant. Rooms, buildings and similar compartments could not, even with difficulty, have germicidal solutions applied to them, for it would be hard to reach all surfaces and then hold such solutions in contact for a sufficient length of time, in order to obtain the desired bactericidal action. In addition, various substances would be injured, if any but a gaseous disinfectant be used as a means of obtaining sterility.

The most practical and yet a suitable and efficient gas for general application is formaldehyde. It is virtually non-poisonous, non-corrosive, non-injurious to all substances, possesses no bleaching effect and still is very effective. There are numerous methods of generating this gas, to be used in practical operations; but this should be noted, when using any of the methods.

Formaldehyde to be effective must be used in an environment, the temperature of which should not be below 60°F., a higher temperature is more preferable. With the gas there should also be an evolution of water vapor, producing a relative humidity of 70 per cent. or over. Unless the conditions be adhered to, it is useless to attempt disinfection with formaldehyde, as otherwise the latter will polymerize, precipitating a solid substance, commonly known as paraform or paraformaldehyde. In all cases where formaldehyde disinfection is performed, all cracks should be hermetically sealed (especially those of doors, windows, etc.) and all drawers and cupboards, containing fabrics and all apparel should be freely opened and the latter spread about to permit sufficient exposure to the gas.

One of the first methods of liberating formaldehyde gas was the method devised by Trilatt, in which he directed the evaporation of the gas from a solution of formalin (a 40 per cent. solution by weight of the gas in water) to which he added from 15 to 20 per cent. of calcium chloride. Such mixture forms a preparation, commonly termed formochloral, which possesses the characteristic property of practically eliminating polymerization. Glycerin has been suggested by Schlossmann, in concentration of 10 per cent. as a substance replacing calcium chloride and hindering polymerization. The apparatus used for the latter and similar solutions consists of a generator, which permits the solution contained therein, when heated, to flow in a fine stream through a copper coil heated to redness by a flame, the gas vapor then passing directly into the room together with some moisture in a superheated and effective condition. Such generators can be operated outside the room to be disinfected.

Similar to the latter and a most efficient method of disinfecting with formaldehyde is the generation of the gas within an autoclave set to blow off at

45 pounds pressure. The apparatus is set up outside the room or compartment to be treated and a hose attached to the autoclave delivers formaldehyde-laden steam through an opening.

Blankets or sheets immersed and saturated with a solution of formalin have been recommended to be hung in and about the room to be disinfected, to allow the liberation of the gas by the heat present. To displace this, some have proposed spraying the room with formalin from a compressed-air or steam atomizer. Neither of these methods is certain as to the liberation of gas and the operator must be careful and work rapidly, as formalin is irritating to the conjunctiva and other mucous membranes.

A most efficient and convenient method used is the liberation of the gas within the room to be disinfected. The method used with universal approval is the permanganate method. Potassium permanganate when mixed with formalin results in an active reaction with the evolution of heat, which in turn causes the evaporation of formaldehyde gas together with water vapor. While some of the gas is actually consumed in the violent reaction, the yield is more than by other methods and preferable because of the quick and sudden evolution of all the gas, together with some moisture.

In practice, 1 quart of formalin is poured over 1 pound of permanganate crystals, using such proportions for each 1000 cubic feet of space. The operator must be cautioned not to throw the permanganate into the formalin, for an explosion will result. On account of the vigorous ebullition and foaming that takes place, high cylindrical vessels should be used, about 10 or 12 inches in height, which possess flared or funnel-like tops, so as to further assure any of the sputtered material from being thrown out. A small 10-quart milk pail answers well for such a purpose.

Numerous modifications have been attempted recently to use other chemicals instead of the permanganate salt. Potassium and more recently sodium dichromate have replaced the latter, due to the cheapness in cost of material. Others have used lime or quicklime, in which method the gas was generated by pouring 1 pint of the solution of formalin over quicklime (from ½ to 1½ pounds) contained in a wide shallow pan, placed in a basin of water.

The dangers and inconveniences experienced with the foregoing methods may be avoided if we use the "so-called" formaldehyde candles or other forms of solid formaldehyde, as the source from which to obtain the gas. The Sherring lamp was the first apparatus, in which solid formaldehyde or paraform was used as a means from which to generate the gas. Heat was obtained from the ignition of alcohol, which in turn decomposed the paraformaldehyde, liberating gaseous formaldehyde. A temperature of at least 275°F. is required for the active generation of this gas from paraform.

All other forms of solid formaldehyde and paraform candles are used the same way, by applying heat to these substances, contained in metal receptacles. Two ounces of paraformaldehyde generates sufficient gas to insure efficient disinfection of a room having a capacity of 1000 cubic feet.

Numerous suitable cabinets have been recently made and are in use by pharmacists, sanitary barber shops and other dealers for the disinfection of shaving, hair, tooth and other brushes. Paraform is here used as the generator and the gas passing through wire shelves can readily come into contact with all articles placed therein.

The value of gaseous formaldehyde as a germicide has from time to time been disputed. This question has, however, been recently settled by a discussion, in which numerous authorities and health officers entered; all favoring a continuance of formaldehyde gas disinfection (see Williams, "Aerial or Gaseous Disinfection," Journ. of the American Pharmaceutical Association, March, 1917, 391).

Sulphur has been extensively used for the generation of SO_2 gas, which is an efficient germicide. In order to be effective, water must be vaporized, since the disinfecting action takes place upon the formation of sulphurous acid by the gas with water. From 3 to 3½ pounds of sulphur should be burned for each 1000 cubic feet of space and the gas be allowed to remain in contact for at least 24 hours. This gas is an active bleaching agent. It is perhaps more used as an insecticide rather than a germicide, a property which is not possessed by formaldehyde. When used for killing insects and vermin water need not be used in conjunction with it, because it is just as efficient for this purpose when dry, and thus will not seriously tarnish or bleach the contents of the room.

Don't attempt to use formaldehyde and sulphur at the same time, the latter as an insecticide and the former as a germicide. Instead of acting as synergists they seem to oppose each other.

Oxygen in its nascent state is an active bactericidal agent. It is this gas that performs the germicidal action when chlorine is used as a gaseous disinfectant. The latter acts only in the presence of moisture, in which the hydrogen of the water molecule unites to form HCl with the chlorine, liberating the nascent oxygen. The latter then does the disinfection. This gas practically is inadequate because of its active bleaching and injurious properties upon all materials.

Bromine has been used both as an insecticide as well as a germicide, but is so poisonous as to be too dangerous for ordinary use.

Hydrocyanic gas is used as an insecticide. It is powerful enough to kill insects but has no effect upon foliage. When used, the trees, foliage and other environment are covered to keep in the vapor and the gas is generated from KCN and dilute HCl, being kept in over night. This gas is rarely used for any purpose, other than the disinfection of foliage, because of its deadly poisonous properties.

For years the apothecary has been directed to have present in the containers of the various drugs, small vials containing chloroform, ether or other such volatile substances, which because of their antiseptic character, are both detrimental to the propagation of bacteria and perhaps more so to the life of the various parasites these drugs harbor. Notable examples of such drugs are ergota linums, etc. The various "waters" of the United States Pharmacopeia may be kept for a greater length of time by adding to such containers a few drops of

Antiseptic		Staphlococcus aureus						Bacillus coli communis				
		In water + 0.7 per cent. peptone		In serum		Concentration which inhibits phagocytosis	Therapeutic coefficient	In water + 0.7 per cent. peptone		In serum		Therapeutic coefficient
		Lethal concentration	Antiseptic potency	Lethal concentration	Antiseptic potency			Lethal concentration	Antiseptic potency	Lethal concentration	Antiseptic potency	
Chloramine-T (content in "available" Cl = 25 per cent.	Concentration of substance........	1 : 2000	8	1 : 250	1	1 : 625	0.4	1 : 2000	8	1 : 250	1	0.4
	Concentration of "available" Cl...	1 : 8000	32	1 : 1000	4	1 : 2500	....	1 : 8000	32	1 : 1000	4	
Eusol (content in "available" Cl = 0.34 per cent.	Dilutions of standard solution....	1 : 13	0.05	1 : 3.25	0.0125	1 : 13	0.25	1 : 13	0.05	1 : 3.25	0.0125	0.25
	Concentration of "available" Cl...	1 : 4000	16	1 : 1000	4	1 : 4000	....	1 : 4000	16	1 : 1000	4	
Dakin's sol. (modified by Daufresne = 0.22 per cent. "available" Cl).	Dilutions of standard solution.....	1 : 9	0.036	1 : 2.25	0.01	1 : 9	0.25	1 : 9	0.036	1 : 2.25	0.01	0.25
	Concentration of "available" Cl...	1 : 4000	16	1 : 1000	4	1 : 4000	....	1 : 4000	16	1 : 1000	4	
Chlorine water (concentration of "Cl")....		1 : 2500	10	1 : 1000 Not sufficient	....	1 : 1500	....	1 : 2500	10	1 : 1000 Not sufficient		
Carbolic acid...........................		1 : 250	1	1 : 250	1	1 : 500	0.5	1 : 500	2	1 : 500	2	1.0
Mercury perchloride.....................		1 : 1,000,000	4000	1 : 10,000	40	1 : 7000	1.4	1 : 1,000,000	4000	1 : 10,000	40	1.4
Iodine (in Ki)*.........................		1 : 10,000	40	1 : 700	2.8	1 : 3500	0.2	1 : 5000	20	1 : 800	3.2	0.2
Brilliant green sulphate................		1 : 10,000,000	40,000	1 : 30,000	120	1 : 2000	15	1 : 130,000	520	1 : 3500	14	1.7
Brilliant green oxalate..................		1 : 10,000,000	40,000	1 : 100,000	400	1 : 7000	14	1 : 200,000	800	1 : 3500	14	0.5
Malachite green oxalate and sulphate......		1 : 10,000,000	46,000	1 : 40,000	160	1 : 7000	6	1 : 20,000	80	1 ; 1000	4	0.15
Crystal violet..........................		1 : 4,000,000	16,000	1 : 400,000	1600	1 : 7000	57	1 : 8000	32	1 : 8000	32	1.1
Ethylhydrocuprein hydrochloride.........		1 : 660	2.6	1 : 2000	8	....	....	1 : 660	2.6	1 : 660	2.6	
Flavine.................................		1 : 20,000	80	1 : 200,000	800	1 : 500	400	1 : 1300	5.2	1 : 100,000	400	200

Lethal concentration = that which suffices to kill organisms as tested by subcultures.

$$\text{Therapeutic coefficient} = \text{ratio } \frac{\text{concentration which reduces phagocytosis by 50 per cent.}}{\text{lethal concentration in serum}}$$

$$\text{Antiseptic potency} = \text{ratio } \frac{\text{lethal concentration of substance in question}}{\text{lethal concentration of chloramine-T in serum (taken as one)}}.$$

*KI in the concentration employed in the solution of Iodine by itself produces no bactericidal action.

chloroform or ether. The small quantity of antiseptic so added is not detrimental or observable either during dispensing or compounding, but yet sufficient to prevent the growth of moulds and other bacteria.

Inasmuch as the retail pharmacist today is called upon to dispense numerous preparations and medicaments as sterile products, it would be advisable to note what medicines require sterilization. These may be grouped as follows:

(*a*) Those preparations intended for intravenous, intramuscular and hypodermic use, which must be absolutely sterile.

(*b*) Drops, lotions, ointments, and other applications to be used for the eye.

(*c*) All preparations to be applied to the bruised or abrased skin and generally in other cases when the latter is broken and medicaments are to be applied thereon.

(*d*) Solutions to be used for inflamed mucous surfaces and for irrigation of various internal organs.

(*e*) All preparations that become mouldy, which in turn may affect their composition, as liquor magnesii citratis (U.S.P.).

Many of the products that the pharmacist is called upon to dispense are extemporaneous preparations, to be dispensed as quickly as possible. It is perhaps true that some of these cannot be made absolutely sterile in the short time available, but all of them can be made as near sterile as is essential for ordinary administration.

Ointments.—All bases and vehicles can be kept sterile indefinitely if the original product when first made be kept in a clean, sterile container in a cool place; and each time a portion is to be removed, a spatula, which has been cleansed with a little alcohol or chloroform, should be used.

Most of the powders entering into the composition of an ointment, if kept in a clean-stoppered container will, with few exceptions, not require any additional means to produce a sterile product.

In compounding, use a clean slab and spatula, which have been previously cleansed with alcohol or chloroform. Place the end product preferably in a collapsible tube, a supply of which can easily be kept on hand, at all times, in a jar containing alcohol, to assure sterility.

Liquid Preparations.—Use sterile containers and stoppers. Use sterile water and have all other solutions sterile if convenient, when these enter into the preparation of any medicinal product which is to be sterile.

If such preparations are not rendered inactive or have their composition otherwise affected, place them either in the autoclave, steam sterilizer or water bath for a sufficient length of time.

In those instances where fractional sterilization is recommended, heating for one period will suffice for all such extemporaneous preparations.

Sterilization by heat may be accomplished by exposure to fire, by hot air, hot water or steam.

Small instruments which can be easily and entirely flamed, without injury, may be conveniently sterilized in this way. Larger instruments, utensils, empty flasks, jars, bottles, tubes and pipettes, are commonly sterilized in a hot-air sterilizer. They can, if necessary, be sterilized by boiling or steaming, but ordinarily, hot air is preferable.

Culture media and pharmaceutical preparations as a whole cannot be sterilized by hot air without destruction.

Hot-air sterilizers are constructed to maintain an even distribution of heat throughout, and are equipped with thermometers. It is a safe rule, especially when sterilizing thick glass and inflammable material, to put the articles in the sterilizer before it has been heated, to elevate the temperature gradually. After sterilization allow the sterilizer and its contents to cool to room temperature before opening the sterilizer.

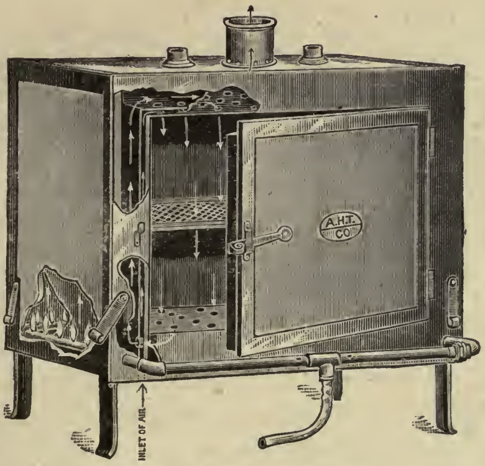

FIG. 4.—LAUTENSCHLAGER HOT-AIR STERILIZER.

Hot air lacks the penetrating power of steam and at the same temperature, hot air is less germicidal than steam. Exposure to a temperature of 150°C. for 1 hour or 200°C. for 30 minutes in the hot-air sterilizer is necessary to affect the destruction of bacteria. Spores frequently withstand this and to destroy them a temperature of 150°C. must be maintained for several hours or 200°C. for 2 hours.

The quickest and most sure method of sterilization is accomplished by means of an autoclave. An autoclave is a vertical or horizontal cylinder, constructed like a boiler. There is a gas burner beneath it for heating, and a water jacket several inches deep at the bottom. A perforated, false bottom, rises above the water. An air-tight door or lid on one end opens to permit loading and unloading. This lid is fastened in place with a number of thumb screws. It is equipped with a dial which registers pounds pressure, and two valves, an exhaust valve and a safety valve. The safety valve is set to blow off steam at 15 pounds pressure. To operate one must first see that sufficient water is in the autoclave (several inches); the substance to be sterilized is then placed in the autoclave and the lid fastened tight with the thumb screws. The exhaust valve is opened and the burner under the autoclave lighted. As the autoclave becomes hot the contained air expands and escapes through the open valve, then steam forms, eventually all the air is expelled and steam begins to escape. When steam comes out of the exhaust valve, it is closed, then the pressure within rises until it registers 15 pounds on the dial. As soon as the pressure reaches 15 pounds the time is noted and exactly 20 minutes later the flame beneath the autoclave is turned out. Gradually the pressure falls as the autoclave cools. It is not opened until the needle on the dial falls to the zero mark. Then the contents of the autoclave are removed, sterile. An exposure to steam under 15 pounds pressure for 20 minutes, kills spores as well as bacteria; it achieves complete sterility.

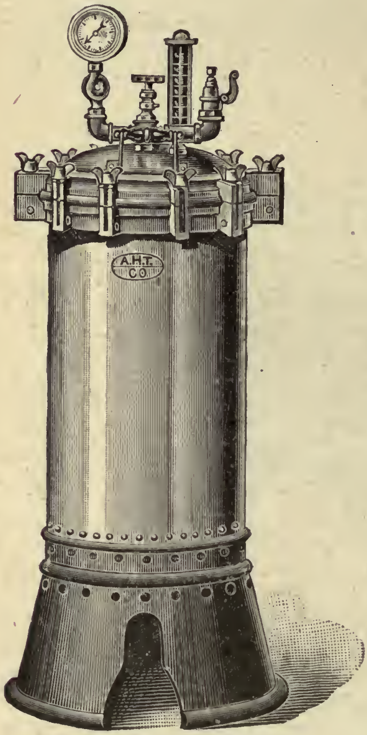

FIG. 5.—AUTOCLAVE.

This is the method of choice for sterilizing water, salt solution, bouillon, agar, milk, camphorated oil, mercury salicylate, mercury sozoiodolate and other substances which are not injured by so high a temperature, 120°C. When an autoclave is not available and a substance suitable for autoclave sterilization must be sterilized at once, make brine by adding common salt to water, place in a water bath or boiler, immerse the substance to be sterilized and boil from 30 to 60 minutes. The addition of salt to water elevates its boiling point close to the temperature of the autoclave at 15 pounds pressure.

Steam sterilization without pressure is usually done in an "Arnold" or similar sterilizer, an apparatus having a square metal pan about 4 inches deep, in the center of which is an upright cylinder about 6 inches in diameter and 6 inches high, that opens into a large cubical compartment. This upper compartment, in which sterilization is affected, has double walls and an inner and an outer door. The apparatus is so constructed that when the pan is filled with water, a flame placed beneath it and steam generated, the steam escapes through the cylinder in the center of the pan into the sterilizing compartment above. Anything that can be sterilized in an autoclave without injury, may be sterilized in the "Arnold" steam sterilizer by steaming it 1 hour each day for 3 consecutive days. This method of sterilizing by short exposure each day, for a number of days, is called **intermittent, discontinuous** or **fractional** sterilization. It is based on the theory that steam at 100°C. will kill all bacteria in 1 hour, but not spores, the spores surviving the first hour of sterilization, develop, in the following hours, into bacteria, and as bacteria, are killed when steamed the second day. The third exposure killing bacteria, developing from spores that might have escaped the first two exposures. For spores to develop into bacteria, conditions favorable to growth must prevail during the periods between steamings. The material upon, or in which the spores are lodged, must contain available bacterial food and the temperature must be about 20°C. to 35°C. It will be seen that intermittent or fractional sterilization is not adaptable to sterilization of instruments, etc.

Quite a number of culture media and many pharmaceuticals undergo chemical changes and are injured by autoclaving, boiling in brine or steaming for an hour. These are sterilized according to the degree of heat they can withstand. Some are placed in the steam sterilizer for ½ hour for 3 consecutive days, others are given 20 minutes or 10 minutes each day for 3 consecutive days. Preparations that cannot be exposed to a temperature of 100°C. for any time without injury, are sterilized by heating them in a water bath at 90°, 80°, 70° or 60°C. from 15 minutes to 1 hour each day for 5 or 6 consecutive days, according to their degree of stability.

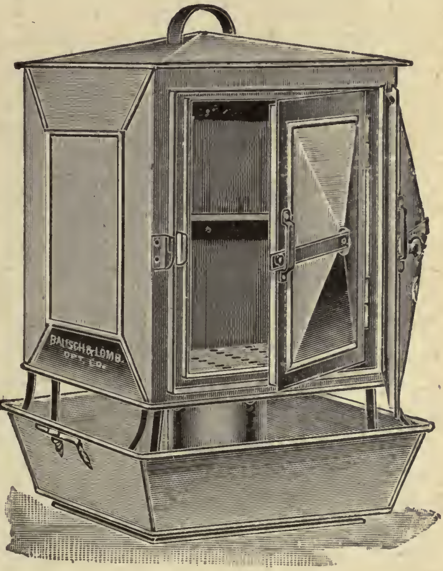

Fig. 6.—Arnold Steam Sterilizer.

Since some spores and a few bacteria can resist temperatures below the boiling point for many hours, to insure the sterility of preparations that cannot be heated above 80° or 90°C., ingredients that may be autoclaved or steamed, are first sterilized that way. Sterile utensils are used throughout preparation and care taken to avoid contamination by air, etc. As an example, let us consider the preparation of litmus lactose gelatin. A saturated aqueous solution of litmus is made, also plain bouillon; these are separately sterilized in autoclave. Gelatin which has been kept sealed in dust-proof packages to prevent possible contamination is dissolved in sterile bouillon, sterile litmus added, and then 1 per cent. of lactose, the lactose having been kept in an air-tight container and weighed out on a sterile watch glass. By means of a sterile test-tube filler, the litmus lactose gelatin is run into cotton-stoppered test-tubes or flakes previously sterilized in a hot-air sterilizer. This litmus lactose gelatin, which cannot withstand steaming for prolonged periods, which has been guarded against contamination and made of sterile ingredients, so far as possible, usually contains few bacteria and fewer spores. It is placed in the steam sterilizer for 15 minutes each day on 3 consecutive days, being kept at room temperature between steamings. In nearly all cases complete sterility is produced.

STERILIZATION TABLES

Hot Air

Material	Temperature	Time, hours
Flasks, bottles, test-tubes, etc., stoppered with cotton or wrapped in paper	150°C.	1 to 3
Flasks, petri dishes, tubes, ampules, pipettes and other utensils, not injured by heat, not stoppered nor wrapped with inflammable material	200° to 300°C.	1 to 2

Talc, 150°C.	1 to 3 hours
Kaolin, 150°C.	1 to 3 hours
Boric acid, 150°C.	1 to 3 hours
Zinc oxide, 150°C.	1 to 3 hours

(Powders should be spread in a stratum not more than ¼ inch thick.)

Autoclave—15 Pounds Pressure for 20 Minutes

Water
Salt solution
Oils
Milk
Potato
Plain bouillon
Plain agar
Glycerin bouillon
Glycerin agar
Mercury sozoiodolate
Mercury Salicylate

Steam (100°C.)

Material	Time, minutes	No. of days
Gelatin	15	3
Bouillon and agar containing sugar	15	3
Sugars	15	3
Litmus milk	15	3
Adrenalin	15	3
Argyrol	15	3
Arsenic	15	3
Atropin	15	3
Caffeine	15	3
Calomel cream	15	3
Gray oil	15	3
Gums	15	3
Mercury benzoate	15	3
Mercury cacodylate	15	3
Morphine	15	3
Paraffin	15	3
Sodium cacodylate	15	3
Stovaine	15	3
Strophantin	15	3
Endo's medium	15	3
MacConkey's bile-salt medium	10	2
Alkaloids, generally	10	2

HOT-WATER BATH (60° TO 80°C.)

Material	Time, hours	No. of days
Blood serum	1	4 to 6
Cacodylates	1	4 to 6
Caffeine benzoate	1	4 to 6
Cocaine	1	4 to 6
Duboisine	1	4 to 6
Ergot	1	4 to 6
Eserine sulphate	1	4 to 6
Glycerophosphates	1	4 to 6
Hyoscine	1	4 to 6
Quinine	1	4 to 6
Physostigmine	1	4 to 6
Scopalamine	1	4 to 6
Strychnine	1	4 to 6
Trypsin	1	4 to 6

Heating at relatively low temperatures (60° to 80°C.) for an hour each day over a number of days is called pasteurization. It insures the destruction of over 90 per cent. of bacteria and attenuation of those not destroyed; so much is accomplished by the first heating. Spores resist this treatment for from 4 to 5 days sometimes. While pasteurization continued for a week usually sterilizes, sometimes it fails to; therefore, pasteurized materials should be tested and examined to prove sterility, before they are used. This is imperative when dealing with quinine preparations intended for hypodermic or intravenous administrations, as quinine may be contaminated with tetanus spores.

Different species of bacteria, even different strains of a single species, vary in their resistance to heat. As heating tends to lessen the therapeutic value of bacterins or bacterial vaccines, the temperature and time of exposure must be varied so as to produce sterilization with the minimum temperature and time of exposure that will accomplish it.

STERILIZATION OF BACTERINS

Organism	Temperature	Time, hours
Typhoid	60°C.	1
Staphylococci	60°C. to 80°C.	1
Streptococci	55°C.	1
Meningococcus	55°C.	1
Gonococcus	60°C.	1
Pneumococcus	60°C.	1
Micrococcus catarrhalis	55°C.	1
Bacillus of influenza	55°C.	1
Bacillus of diphtheria	60°C. to 70°C.	1
Bacillus of friedländer	55°C.	1
Bacillus of dysentery	55°C.	1
Bacillus of paratyphoid	55°C.	1
Bacillus coli	55°C.	1
Bacillus pestis	55°C.	1
Spirillum of cholera	55°C.	1
Sensitized vaccines	55°C.	½

CHAPTER VI

CULTURE MEDIA

Culture media are sterile fluids and solids upon which bacteria are cultivated to determine the character of their growth, to study and classify species, to isolate some organisms from others, to detect the presence, number and kind of bacteria in water, milk, etc., and to obtain large numbers of certain organisms for the preparation of diagnostic and therapeutic agents, such as bacterial vaccines.

For convenience of description, culture media may be divided into two classes, natural and artificial.

NATURAL CULTURE MEDIA

Fluid

- Milk
- Whey
- Blood serum
- Serous fluids
- Eggs
- Ox bile
- Urine

Solid

- Blood serum
- Eggs
- Potato

ARTIFICIAL CULTURE MEDIA

Fluid

- Bouillon
- Lactose bouillon
- Glucose bouillon
- Saccharose bouillon
  - With or without litmus
- Other sugar bouillons
- Glycerin bouillon
- Ox-bile bouillon
- Dunham's solution
- MacConkey's bile-salt-medium
- Trypsinized-peptone-water

Solid

- Agar
- Lactose agar
- Glucose agar
- Saccharose agar
  - With or without litmus
- Other sugar agar media
- Glycerin agar
- Blood-smeared agar
- Endo's agar
- Russell's agar
- Malachite-green agar
- Gelatin media
- Petroff's medium

Some bacteria grow luxuriantly on a variety of media, others will only grow on a few substances especially adjusted to their requirements. Hence, we have a number of culture media, some used for the cultivation of bacteria in general and others for the cultivation of one or several species.

PREPARATION OF CULTURE MEDIA

Litmus Milk.—Fresh, sweet, cow's milk, which contains not more than 3 per cent. fat:

1. Add aqueous sol. of litmus, sufficient to give a faint blue tint.
2. Sterilize in Arnold steam sterilizer, 1 hour each day for 3 consecutive days.

Plain Bouillon (Meat extract broth).

1. Beef extract (Liebig's), 2 Gm.
2. Beef peptone (Witte's), 10 Gm.
3. Sodium chloride, 5 Gm.
4. Water, 1000 cc.

Make a paste of the peptone; then dissolve it in water, add the salt and beef extract. Boil and stir for ½ hour, make up for evaporation by the addition of water, again bring to boiling point. Test for alkalinity or acidity and properly adjust the reaction. Filter while hot and again when cool. Sterilize in autoclave.

Plain Bouillon (Meat infusion broth).

1. Minced lean meat (beef or veal), 500 Gm.
2. Water, 1000 cc.

Place in ice box in shallow dish for 24 hours. Skim off fat, filter through linen and express juice contained in the meat.

Boil for 2 hours, filter through linen and boil again for 1 hour. Add water to make up for loss by evaporation, add peptone 10 Gm. and sodium chloride 5 Gm., boil for ½ hour, make up for evaporation, adjust reaction, filter while hot and again when cool. Sterilize in autoclave.

Sugar-free Bouillon.—Make meat infusion broth, introduce bacillus coli and incubate for 24 hours at 37°C., boil for ½ hour, filter while hot and again when cool. Adjust reaction and sterilize.

Sugar Bouillon.—Various sugars are added to plain bouillon or sugar-free bouillon to study fermentation. Glucose, lactose and saccharose are most commonly used. ½ to 2 Gm. to 100 cc., ½ to 2 per cent.

Bouillon containing sugar is often tinted a faint blue by adding litmus; this is to act as an indicator of acid production.

Culture media containing sugars are sterilized in the Arnold steam sterilizer (100°C.) 20 minutes each day for 3 days.

Glycerin Bouillon.

1. Plain bouillon, 100 cc.
2. Double distilled glycerin, 6 cc.

Sterilize in autoclave.

Calcium Carbonate Bouillon.—Sterilize powdered calcium carbonate or marble chips in hot-air sterilizer at 150°C. for 2 hours.

1. Plain bouillon (meat infusion), 100 cc.
2. Calcium carbonate, 1 Gm.
3. Glucose, 1 Gm.

Sterilize in steam sterilizer at 100°C. 20 minutes each day for 3 days.

Dunham's Solution (Peptone-salt-sol.).

1. Peptone (Witte's), 10 Gm.
2. Sodium chloride, 5 Gm.
3. Distilled water, 1000 cc.

Boil 15 to 30 minutes, add water to make up for evaporation, filter while hot and again when cold.

Sterilize in steam sterilizer at 100°C. 20 minutes each day for 3 days.

Trypsinized Peptone-water, (Rivas).

1. Dissolve 10 Gm. peptone (Witte's) with gentle heating in 200 to 300 cc. of water.
2. Dissolve 0.5 Gm. trypsin in 20 cc. of water by shaking and gentle heating (less than 40°C.).
3. Mix and digest for 2 to 3 hours at 38°C., stirring gently every 15 minutes.
4. Adjust reaction to neutral, add water to make 1000 cc., boil, filter, tube and sterilize in an autoclave.

This medium is superior to Dunham's for detection of indol production.

Ox-bile Bouillon.

1. Plain bouillon, 100 c.c.
2. Ox-bile, 100 cc.

Sterilize in steam sterilizer 20 minutes each day for 3 days.

Bile Medium (Buxton and Coleman's).

1. Ox bile, 900 cc.
2. Glycerin, 100 cc.
3. Peptone, 2 Gm.

Sterilize in steam sterilizer 20 minutes each day for 3 days.

Hiss Serum Water Medium.

1. Beef-blood serum, 100 cc.
2. Distilled water, 300 cc.
3. Glucose or other sugar, 4 Gm.
4. Sat. aqueous sol, litmus q. s. to make blue.

Sterilize in steam sterilizer 20 minutes each day for 3 days

MacConkey's Bile Salt Medium.

1. Sodium taurocholate, 0.5 Gm.
2. Peptone, 2 Gm.
3. Water, 100 cc.

Steam for 2 hours, filter while hot, place in ice box over night; filter, add 0.25 cc. of 1 per cent. aqueous sol. neutral red (freshly prepared) and sugar ½ or 1 per cent. Glucose, dulcite or adonite ½ per cent.—other sugars 1 per cent. Sterilize in steam sterilizer 10 minutes each day for 2 days.

Gelatin Medium.

1. Plain bouillon, 100 cc.
2. Gelatin (best French leaf), 10 to 30 Gm.

Break up gelatin, allow it to soak in the bouillon for 5 minutes; then, heat and stir until dissolved and adjust reaction.

Sterilize in steam sterilizer for 15 minutes each day for 3 days.

Modifications of gelatin, such as glucose, lactose, saccharose, or other additions and coloring with litmus, are made by adding from 1 to 2 per cent. of the sugar required and tinting is accomplished by adding sufficient sat. aqueous solution of litmus to give a faint blue tint.

Agar Medium.

1. Plain bouillon, 100 cc.
2. Agar, 1 to 3 Gm.

Either shredded or powdered agar may be used, but for most purposes the former is preferable. If shredded agar is used it should be cut into small pieces before putting it into the bouillon.

Boil and stir until agar is dissolved, add water to make up for the loss by evaporation, adjust reaction and filter in a warm place, as agar solidifies at about 35°C.

Sterilize in autoclave.

When sugars alone, or sugars and litmus, are added to agar, it is done the same as directed for bouillon and gelatin, and sterilized 15 minutes each day for 3 days.

Glycerin Agar Medium.—Prepare agar as directed; then, add 6 per cent glycerin.

Sterilize in autoclave.

Russel's Double Sugar Agar Medium.

1. Plain agar (3 per cent.), 100 cc.
2. Lactose, 1 Gm.
3. Glucose, 0.1 Gm.

Sat. aqueous sol. litmus violet tint.

Sterilize in tubes in steam sterilizer 15 minutes each day for 2 days and slant.

Endo's Medium.

1. Nutrient agar (3 per cent.), 1000 cc.
2. Lactose, 10 Gm.
3. Filtered sat. sol. basic fuchsin in 95 per cent. alcohol, 3 cc.
4. Sodium sulphite solution (10 per cent.), 30 cc.

Liquefy agar in Arnold steam sterilizer. Dissolve in it the lactose. Test reaction. Best results are obtained where the medium is neutral or just slightly alkaline to litmus paper. Add exactly 3 cc. fuchsin solution and 30 cc. sodium sulphite solution (or, the sod. sulph. being hygroscopic, you may add instead of 30 cc. of the 10 per cent. sol., 3 cc. of sat. liquid usually found in even tightly stoppered bottles).

The resulting mixture is quite red when hot but loses its color on cooling. After tubing, sterilize for 20 minutes each day, for 3 days, in Arnold steam sterilizer.

Loeffler's Malachite-green Agar Medium.

1. Meat infusion bouillon, 500 cc.
2. Distilled water, 500 cc.
3. Neutralize.
4. Normal hydrochloric acid sol., 7.5 cc.
5. Shredded agar, 30 Gm.
6. Boil until agar dissolves, then neutralize with normal sodium hydrate.
7. Add normal sodium carbonate sol., 5 cc.
8. Place in steam sterilizer for 2 hours; then, add—
9. 10 per cent. aqueous sol. nutrose, 100 cc.
10. Sterilize in steam sterilizer 15 minutes each day for 2 days.

When ready to use, liquefy and add between 2 cc. and 3 cc. of a 2 per cent. aqueous sol. of malachite green (Hochst 120) to each 10 cc. of medium.

Conradi and Drigalski's Medium.

Minced beef, 750 Gm.
Water, 1000 cc.

Put in shallow dish, in ice box, over night. Skim off fat, boil for 1 hour, filter, add water to bring volume to 1000 cc.

Add—

Peptone (Witte's),	10 Gm.
Nutrose,	10 Gm.
Calcium chloride,	5 Gm.—

boil; add 30 Gm. of agar, boil until agar is dissolved; make faintly alkaline to litmus paper; autoclave for 1 hour; filter through paper and autoclave for ¼ hour.

Add—

Litmus solution (Kahlbaum)	150 cc.
Lactose	15 Gm.

Sterilize in Arnold steam sterilizer for ¼ hour.

Add—

Hot, sterile, 0.1 per cent. solution of crystal violet (B. Hochst), 10 cc.

This medium favors growth of the typhoid bacillus and inhibits the colon. On it typhoid colonies are transparent, blue-white, and cause no change in color of medium. Colon colonies are red and opaque and the medium immediately surrounding them is turned from blue to red.

Blood-smeared Agar Medium.—Make plain agar, put in tubes, sterilize in autoclave and slant. When the agar solidifies, wash a finger with soap and hot water, alcohol, 1:1000 bichloride sol., sterile water, then dry with sterile towel. Prick the finger with a sterile needle, catch the blood in a sterile platinum loop and smear it over the entire surface of the agar.

Blood Agar Medium.—Etherize a rabbit, shave and asepticize its chest, take a 10-cc. or 20-cc. sterile syringe and needle and withdraw blood from heart, empty blood into a sterile bottle containing sterile glass beads and shake vigorously until defibrinated. Bring temperature to 45°C. in water bath. Have tubes containing 10 cc. sterile, plain agar, liquefied, and at a temperature of 45°C. With a sterile pipette add 5 cc. of blood to each tube of agar, mix blood and agar and slant.

Blood Alkali Agar Medium.—Obtain ox blood in slaughter house. Collect in small sterile bottles containing glass beads (100 cc. to 300 cc., cap bottles), shake until defibrinated.

Mix equal volumes of blood and normal sodium hydrate solution. Sterilize in steam sterilizer 15 minutes each day for 2 days.

Add 1 part of this alkaline blood to 7 parts sterile plain agar, place in tubes and slant. Do not have cotton plugs any tighter than necessary.

Loeffler's Blood-serum Medium.—Blood serum (calf's or lamb's), 3 parts.
Glucose bouillon, 1 part.

Put in tubes, place in serum sterilizer. Heat at 57°C. 1 hour each day for 6 days, then at 70°C. 1 hour each day for 2 days. Or heat in Arnold steam sterilizer at 80°C., 1 hour each day for 3 days.

A Substitute for Ordinary Blood Serum.[1]—"Add from 10 to 15 cc. of 1 per cent. glucose bouillon to the white and yolk of one egg, make smooth mixture in a mortar and place in tube. Inspissate and sterilize as for ordinary serum slants."

"When this medium is to be used for culturing tubercle bacilli, add about 1 cc. of glycerin bouillon to each tube before final sterilization in the autoclave. The cotton plugs should be paraffined to prevent drying of the slants in the incubator."

Egg Medium.—Select a clean, fresh egg (not more than 24 hours old) that has a perfect shell, no cracks; shake to mix white and yolk, wash with warm sterile water, then with warm 1:500 bichloride solution and again with sterile water, wipe dry. To inoculate, pierce shell with sterile needle and introduce bacteria with platinum loop, seal opening with wax. If anaerobic culture is desired, coat egg with paraffin.

Dorset's Egg Medium.—Selected clean fresh eggs with perfect shells. Wash with sterile water, then with 5 per cent. phenol and finally with sterile water. Break with a sterile knife and empty the eggs into a sterile flask.

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[1] Stitt, "Practical Bacteriology," 3d edition, p. 25.

Add 10 per cent. (by weight) of sterile distilled water. Mix. Filter through gauze, tube, slant and sterilize in steam sterilizer at 70°C. for 2½ hours.

In emptying eggs from shell care must be taken to prevent the eggs from contact with the outside of the shell or fingers; when mixing with water and tubing, bubbling must be avoided.

Potato Medium.—Take a smooth, new potato, wash in water (hot), hot 1:500 bichloride; then, in several changes of hot water. Cut cylindrical pieces about 1½ inches long and about the same diameter as the tubes in which they are to be placed. Split each cylinder diagonally in two through long axis, place each piece in a tube with the thick end at the bottom and add about ¼ inch of distilled water.

Sterilize in autoclave.

Petroff's Medium.

2 parts egg (whites and yolks).

1 part meat juice.

Gentian violet in 1:10,000 dilution.

Meat juice—infuse 500 Gm. of veal in 500 cc. of 15 per cent. sol. glycerin in H_2O for 24 hours and strain off juice.

Eggs—sterilize shells with 20 per cent. carbolic acid and break into sterile beaker and mix well. Then filter through gauze, adding 1 part of the infusion by volume.

Gentian violet—add sufficient 1 per cent. alcoholic gentian violet to make a dilution of 1:10,000.

Tube and sterilize in steam sterilizer or inspissate for 3 successive days, 80°C. for 1 hour each day.

Modification of Petroff's Medium.—(By Williams-Burdick).

1. **Egg-white Solution.**—This is made by diluting the egg white with 10 parts of distilled water and thoroughly shaking. The fluid is opalescent and contains numerous whitish flakes. To clear it, pass it through a thin layer of cotton and then heat to 100°C. to hasten precipitation. It is then filtered through paper.

2. **Egg-yolk Solution.**—The yolks are diluted with 10 parts of water and well stirred. The cloudy emulsion is clarified by adding normal sodium hydroxide. Too much hydroxide is harmful and therefore complete solution of the yolk is not desirable. The emulsion should be slightly turbid. To attain the proper degree of turbidity, 1 cc. of normal NaOH is usually added to each 100 cc. of emulsion. This is not a constant amount, however, because some yolks will be completely dissolved by less than half this amount of alkali. The solution is heated to 100°C. and filtered.

3. **Meat Infusion.**—500 Gm. of finely chopped lean veal are covered with 1 liter of water containing 15 per cent. glycerin, allowed to infuse for 24 hours and filtered; 5 Gm. NaCl are added and the infusion heated to boiling. It is again

filtered and then rendered plus 1 per cent. alkaline. With the above solutions, the medium is made as follows:

Place 300 cc. of the 10 per cent. egg-white solution in a liter flask; 300 cc. of the 10 per cent. egg-yolk solution in another flask, and 400 cc. of the meat infusion, to which is added 15 Gm. of powdered agar-agar, in a third flask. These are then sterilized in the autoclave at 15 pounds pressure for 15 minutes. They are removed from the sterilizer and, while hot, 1 cc. of a 1 per cent. alcoholic solution of gentian violet is added to the broth-agar. The contents of this flask are now poured into that containing the egg white and then the egg yolk is added. The whole is poured back and forth from this flask to another so as to insure thorough mixing, and then it is tubed and slanted. The tubes are left in slanted position for about 72 hours, at room temperature, until the contents are well set, and the tubes sealed with corks or paraffin.

REACTION OF CULTURE MEDIA

The most common organisms which are both saprophytic and pathogenic flourish on media either slightly acid, neutral or slightly alkaline in reaction. Many of these grow most luxuriantly in a medium having a reaction indicated as slightly alkaline when tested with litmus paper (turn red paper slightly blue, do not turn blue paper red). For general purposes, media tested with litmus paper and adjusted to give a slightly alkaline reaction, do well.

Plain bouillon, agar and gelatin media, when made, are usually acid to litmus paper. When it is necessary to add acid or alkali, solutions of hydrochloric acid or sodium hydrate are used.

Although this easy method of testing and adjusting the reaction of culture media is ordinarily satisfactory, it has faults that make more accurate methods preferable, at least for some purposes. The observations of different workers are comparable only when the culture media they use is uniform in reaction. This is especially true and important in culturing milk, water, sewage and foodstuffs to determine the number of bacteria contained. For this reason the American Public Health Association has recommended the following method of testing and adjusting the reaction of culture media:

Reagents—freshly prepared ½ per cent. solution of phenolphthalein in 50 per cent. alcohol—indicator.

Normal, tenth normal and twentieth normal solutions of hydrochloric acid and sodium hydrate.

Place 5 cc. of culture medium in porcelain dish, add 45 cc. distilled water, boil for 3 minutes, add 1 cc. of phenolphthalein (indicator); if acid; the contents of the dish show no change of color; if alkaline, turn red. Usually the reaction is acid; in this case tenth normal NaOH is added, drop by drop, from a graduated burette until the neutral point is reached; the amount of NaOH required to neutralize is recorded, and the experiment repeated, using twentieth normal NaOH.

Should the contents of the dish turn red when the phenolphthalein is added it is alkaline and titration would be done with $\frac{N}{10}$ and $\frac{N}{20}$ HCl.

For preparation of synthetic media, see "Preliminary Report on Synthetic Media," C. J. T. Dorland, Journal of Bacteriology, 1916, vol. i, No. 2, p. 135.

During titration the contents of the dish should be constantly stirred. When NaOH is being added, as each drop falls from the burette it causes a bright rose red to appear, which varnishes on stirring, until the neutral point is reached.

When the contents of the dish show a faint, but distinct pink color, that does not disappear on stirring and heating, the neutral point has been reached.

Having neutralized a measured sample of culture medium with a measured amount of acid or alkali, it is easy to compute just what quantity of one or the other need be added to the bulk of medium to produce any desired degree of acidity or alkalinity—*e.g.*, if we are using a $\frac{N}{10}$ NaOH solution and find that the neutral point has been reached when 2 cc. of it have been dropped into the dish containing 5 cc. of the culture medium, then to neutralize 100 cc. of the culture medium, twenty times as much $\frac{N}{10}$ NaOH would be required, or 40 cc. of $\frac{N}{10}$ NaOH. As 1 cc. of $\frac{N}{1}$ NaOH is equivalent to 10 cc. of $\frac{N}{10}$ NaOH, then 4 cc. $\frac{N}{1}$ NaOH would neutralize 100 c.c of the culture medium.

In establishing a standard for this method of titration the plus sign is taken to indicate acidity, the minus sign to indicate alkalinity

Culture media having a reaction that would require the addition of 1 cc. of normal sodium hydrate to each 100 cc. of media to neutralize it is said to have a reaction of +1. In other words, +1 indicates that the media is so acid that 1 per cent. of normal sodium hydrate would be required to neutralize it.

For general purposes and when the number of bacteria per cubic centimeter is to be determined in milk, foodstuffs, water, sewage, etc., +1.5 is the standard reaction.

As a matter of fact when standard peptone, sodium chloride, meat extract or meat infusion are used in making culture media, when completed, the reaction nearly always falls between +0.5 and +1.5. If the reaction is found to lie between these limits no attempt should be made to adjust it. If it falls outside these limits it should be adjusted to +1.5.

The growth of bacteria is largely influenced by variations in the titratable acidity of culture media. Some organisms are much more sensitive to variations in this than others, and bacteriologists since the days of Pasteur have given careful attention to this fact. Recent investigations have shown that in some instances, probably in many, the hydrogen ion concentration is equally as important as the titratable acidity of media. At the present time no method of measuring or adjusting this is in general use. Undoubtedly, in the near future this will be done. The student is recommended to read Clark and Lubs monograph on this subject, which is the clearest and most informative presentation of the subject. "The Colorimetric Determination of Hydrogen Ion Concentration and Its Applications in Bacteriology," Journal of Bacteriology, vol. ii, No. I, January, 1917.

FILTRATION OF CULTURE MEDIA

Culture media must be clear. This is usually accomplished by filtration.

Bouillon needs to be passed through several thicknesses of filter-paper, sometimes repeatedly, while it is hot and again when cool. Gelatin and agar are cleared by one passage through a single thickness of filter-paper. Filter-papers should be moistened with water and allowed to stand a few minutes before media is poured on them.

If the white of egg is added and the media then boiled for 5 minutes before filtration, much of the suspended matter is caught in the coagulum that forms and less filtration is required. At least one egg to every 1000 cc. of media should be used, the whites well beaten and then thoroughly mixed with the media by stirring.

When egg has been added gelatin and agar can be cleared by filtering through cotton, provided it is done carefully. A piece of gauze should be placed in a funnel, upon this a piece of cotton about ½ inch thick, with all the fibers running in one direction, should be placed. Upon this place a second piece of cotton the same thickness, with its fibers at right angles to those of the bottom layer. Moisten with water and smooth the edges to the side of the funnel; then, gradually run culture media upon it so as to prevent the media from escaping between the cotton and side of funnel. Filtration through cotton is quicker than through paper.

Blood serum to be used in the fluid state and other media that cannot be heated are both cleared and sterilized by filtration through unglazed porcelain tubes.

CONTAINERS FOR CULTURE MEDIA

Media kept in bulk are placed in flasks of from 100 cc. to 1000 cc. capacity; otherwise they are kept in test-tubes. These containers should be made of alkali-free glass, be clean and clear. They are plugged with cotton and sterilized in a hot-air sterilizer before use. The plugs are made of clean non-absorbent cotton. A smooth piece of cotton is placed across the mouth of a tube and pushed in with a pencil or similar instrument; the resultant plug will be tight or loose, according to the size of the piece of cotton, and this must be regulated so that the plug, extending into the tube from ½ to 1 inch, will be firm enough to permit lifting of the tube by grasping the cotton and still not so tight that it cannot be replaced with ease when removed. The cotton plug is made to extend about ½ inch above the top of the tube so that it can be readily removed and held without touching the portion that enters the tube.

Media in cotton-plugged containers tend to dry out, the smaller the bulk, the more rapidly does it dry out.

When necessary this is guarded against by placing rubber caps over the top of the tube and plug or by dipping the top of the tube and plug in melted paraffin.

Culture media keep best when placed in a clean refrigerator.

Should the portion of a tube that the plug comes into contact with be wet with culture media, more or less cotton adheres when the plug is removed, interfering with the introduction and removal of bacteria. This is avoided by filling the tubes with a jacketed test-tube filler constructed to guard the upper part of the tube from contact with media.

After media has been run into tubes, they are never tilted, slanted or shaken to a degree that would send the media into contact with the plugs.

When using tubes 5 or 6 inches long they are usually filled to a depth of about 2 inches with liquid media, such as bouillon, about the same with solid media intended for plating and stab cultures.

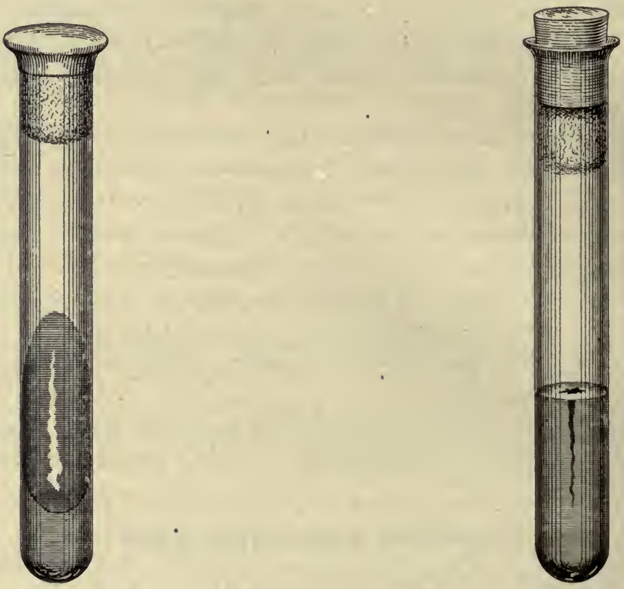

Fig. 7.

Smear-culture. This tube shows the rubber cap used to prevent drying.

Stab-culture. A rubber stopper may be used to prevent drying.

To obtain surface growth on solid media in test-tubes, immediately after sterilization, while the media is still liquid, the tubes are slanted and permitted to remain so until the media solidifies; these tubes should contain such a quantity of media and be slanted at such an angle that when solid the media will entirely fill the bottom ½ inch of the tube, the upper portion being at least an inch below the plug.

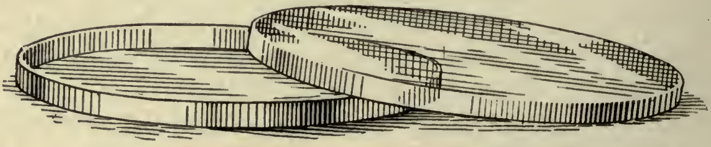

Fig. 8.—Petri Dish (*MacNeal*).

Covered glass dishes—Petri dishes—are frequently employed when a broad surface growth on solid media is desired. They are shallow and intended to contain a comparatively thin layer of culture media, so that media becomes dry in them after several days. Consequently, Petri dishes only have media placed in them at the time one intends to plant bacteria.

The media intended for use in dishes is stored in flasks or tubes. When a plate is to be planted, a flask or tube of sterile media is liquefied in a water bath and poured into a sterile dish; when solid the substance to be planted is dropped on the surface or streaked over it with a sterile platinum wire or glass rod. Another method is to liquefy a tube of culture media, cool it to 40°C., drop the substance to be cultured into it, mix by turning the tube upside down several times, and then pour the contents of the tube into a Petri dish. As a precaution against contamination the cover of a Petri dish is lifted as little as possible and media is not allowed to run over the edges. Dishes are not moved nor placed in an incubator until the contents have solidified.

CULTURE TECHNIQUE

Liquids containing bacteria that are to be cultured are transferred to tubes of culture media with sterile glass pipettes or with loops of platinum wire; solidi and macroscopic masses of bacteria are commonly handled with loops of plats num wire except when stab cultures are to be made, then a straight piece of wire or a needle is used.

When a tube of culture media is to be planted it is desirable to have the air as still and as free of dust as possible, windows and doors are closed and one avoids breathing directly upon or over exposed surfaces of media. The cotton plugs are removed from flasks or tubes containing culture media by grasping the plug between the little finger and palm of the hand, or between the other fingers, care being taken to avoid touching the inside of the tube. While the plug is held between the fingers, that part which enters the tube must not come into contact with anything. Just before replacing the plug it is well to pass it through a flame to burn off any organisms that may have settled on it. The less frequently tubes are opened and the shorter the time the plug is out of the tube, the less danger of contamination. After removing the plug, the open end of the tube is flamed before introducing or removing bacteria; it is again flamed before replacing the plug.

If a pipette is used to inoculate media its contents are dropped into or upon the media without bringing the pipette into contact with the media or the upper portion of the tube. When inoculating liquid media with platinum loop, the loop is held so that it does not touch the side of the tube until submerged in the media, then it is given a quick turn and removed with the same care as when inserted.

Material containing bacteria to be cultivated on the surface of solid media (either slants in tubes or plates) must be gently dropped, smeared or streaked across the surface to avoid breaking the smooth surface.

To determine the character of growth beneath the surface, to observe gas formation and other phenomena, what are known as *shake cultures* are sometimes made in solid media, usually in gelatin. A tube of media is liquefied and cooled to 40°C., the bacteria are put in it and the tube shaken to distribute them, it is then solidified.

Stab cultures made in solid media, usually gelatin or agar, are made by thrusting a needle or straight platinum wire coated with bacteria through the long axis of a tube of media nearly to the bottom.

Gas formation by bacteria is best observed by cultivating in liquid media in *fermentation tubes*, tubes so constructed that displacement of fluid by generated gas is easily discernible.

The best form of fermentation tube consists of an upright cylindrical tube, about 5 inches long and 3/8 inch in diameter, closed at the top and connected at the bottom by a narrower short U-tube to a spherical chamber, of about 10 cc. capacity, which has an opening at the top. These fermentation tubes are plugged with cotton and sterilized in a hot-air oven. The media to be used in them is best handled if tubed, sterilized and stored in test-tubes; each test-tube containing sufficient medium to completely fill the upright arm and one-half of the spherical portion of a fermentation tube.

When a test is to be made the suspected material is first placed in the fermentation tube and the culture medium then poured into the bulb. By tipping the fermentation tube the suspected substance and culture medium mix and flow into the upright arm. Every bubble of air must be displaced from the upright portion of the fermentation tube, so that the formation of gas in the tube may be recognized, as it accumulates at the top of the upright arm.

Indol.—Some bacteria produce indol (C_8H_7N) when cultured on appropriate media, and this fact is taken advantage of in the identification and differentiation of certain organisms. It must be remembered that an organism, like the colon bacillus, which ordinarily produces indol, may at times temporarily lose its ability to do so.

Dunham's solution has been largely used as a culture medium favorable to indol production, but the Trypsinized peptone-water of Rivas seems superior.

The organism to be tested is planted in a test-tube containing Dunham's or Rivas' medium and incubated at 37°C. If only one tube is planted it is tested for indol after 48 hours of incubation. Sometimes sufficient indol is produced in 6, 12 or 24 hours to be detected and hence when the determination is desired as early as possible, it is expedient to plant several tubes and examine one 6 hours, another 12 hours, another 24 hours, and, finally, one 48 hours after incubation.

The presence of indol is disclosed by adding 5 to 20 drops of a 0.2 per cent. solution of potassium nitrite, shake the tube, then add slowly an equal quantity of a 25 per cent. solution of pure sulphuric or hydrochloric acid. If indol is present the culture medium turns red, immediately after the addition of acid, or several minutes later.

For an elaborate discussion of indol production by bacteria, see "Studies on Indol," Rivas, D., Centralblatt für Bakteriologie, Parasitekunde und Infektionskrankheiten, Orig., 1912, lxiii Bd.

Plating.—The cultivation of bacteria in broad, shallow, circular, covered glass dishes (Petri dishes) is referred to as plating. The advantages of this method are the possibility of procuring discrete colonies when quantities are planted that would give a confluent growth in tubes, greater facility of studying the appearance of individual colonies as they occur on the medium, greater possibility of removing a single colony from the medium with a platinum loop without contaminating it by contact with others. Therefore, when substances contain several species of organisms and it is desirable to isolate one or more of them, plating is usually the most convenient method; also, when it is desirable to determine the number of bacteria per cubic centimeter in a substance, plating usually affords the best means of so doing.

Most bacteria associated with disease grow best or only grow at or near the temperature of the human body, hence cultures are usually placed in an incubator at 37°C. to facilitate bacterial growth. Saprophytic bacteria are usually incubated at room temperature or at 20°C. or 25°C.

Aerobic bacteria, those that require oxygen to develop, grow well in bouillon and on the surface of solid media in tubes or flasks stoppered with cotton plugs. Sufficient oxygen percolates through the cotton when the tubes are kept in a room, closet or incubator where the atmosphere is air.

Anaerobic bacteria, those that grow only in the absence of free oxygen, are usually planted in media containing 1 to 2 per cent. of glucose, because glucose is a reducing agent and free oxygen does not exist in media containing it.

When better means are not available anaerobes can be cultivated by taking tubes half-full of solid glucose agar, making deep stab inoculations in the center of the media, and covering the surface with an inch or more of sterile liquid petrolatum.

Anaerobic cultures are usually made by placing the inoculated tubes or Petri dishes in air-tight jars, from which the oxygen is removed either by extraction, displacement or absorption, or a combination of these methods. For extraction and displacement specially devised jars, fitted with taps and stop-cocks are required. Exhaustion is achieved by pumping out the air and leaving a vacuum. Displacement is achieved by introducing hydrogen through one tap while the other is open so that air and oxygen pass out as hydrogen enters. When the atmosphere is entirely devoid of oxygen the stop-cocks are closed. Hydrogen is generated for this purpose in a Kipp's apparatus, from sulphuric acid and zinc. It is passed, in the order mentioned, through three wash bottles; the first containing 10 per cent. lead acetate solution, the second 10 per cent. silver nitrate solution and the third a 10 per cent. solution of pyrogallic acid in 10 per cent. sodium hydrate solution. It is then delivered into the jar containing culture tubes or plates.

Absorption of oxygen does not require special apparatus, any jar having a ground-glass stopper or screw cap that can be made air-tight will do. The tubes or plates are placed in the jar, on a support that elevates them an inch or more above the bottom. About ½ inch of pyrogallic acid is placed in the bottom of the jar, potassium hydrate solution is poured upon it and the jar quickly sealed. Potassium pyrogallate is formed and it absorbs the oxygen. To 2 to 4 Gm.

of pyrogallic acid add 109 Gm. of potassium hydrate dissolved in 145 cc. of water. Should a ground-glass-stoppered jar be used, the ground surface should be anointed with vaseline to insure an air-tight joint, and for the same reason washers are used on screw-top jars.

Quantity of material to be planted on or in culture media to obtain a growth is governed by a number of factors, among which are the following:

Some organisms grow luxuriantly when planted on culture media, regardless of whether taken directly from tissue (sputum, pus, blood, urine, etc.) or from previous laboratory cultures; others grow luxuriantly after several transplants from culture media to culture media, but show very scant growth, and that irregularly, when first transplanted from tissue or exudate to culture media; some never grow abundantly and are irregular in growing, no matter how often they are transplanted from media to media and some grow abundantly on certain media and poorly on other media. For these reasons the quantity of any substance planted on media to obtain a culture varies according to the number of organisms contained in the substance, the peculiarities of the particular organism to be cultured and the medium upon which it is planted, also the quantity of growth desired.

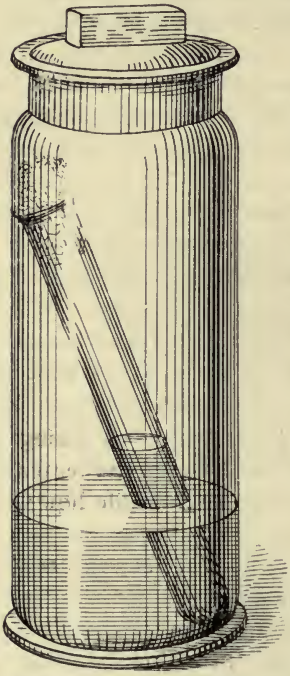

Fig. 9.—Arrangement of Tubes for Cultivation of Anäerobes by Buchner's Method (*MacNeal.*)

To determine the number of bacteria per cubic centimeter contained in any substance, for the isolation of a single organism from a mixture and for future cultural and biological studies of bacteria, one must obtain a growth in which the colonies remain discrete and far enough apart to permit the removal of a single colony without coming into contact with others—20 to 200 on a Petri dish.

When dealing with a substance rich in bacteria that grow abundantly—as feces containing colon bacilli—the almost invisible amount that adheres to a small platinum loop gently touched to it, is sufficient to plant one or several tubes or plates and produce an abundant growth.

Very many organisms, which ordinarily grow abundantly on numerous media, at times fail to grow at all when transplanted; such failure to grow is a more frequent occurrence when organisms difficult to cultivate are dealt with and hence it is always advisable to plant two, three or more tubes or plates, not one.

CHAPTER VII

STAPHYLOCOCCI

Staphylococci are commonly present in air, dust and soil, frequently in the mouth and intestinal tract of healthy as well as diseased people. Almost constantly upon the scalp, hands and other portions of the body surface, in milk and foodstuffs.

Many saprophytic non-pathogenic and saphrophytic and pathogenic species of staphylococci have been isolated and described; most of the latter have very rarely been found associated with disease in man and of these some appear to have a selective affinity for certain organs of the body, similar to the predilection for certain organs, according to Rosenow, shown by streptococci.

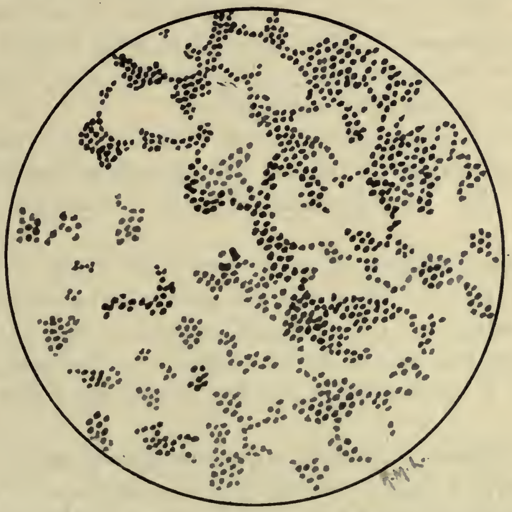

Fig. 10.—Staphylococcus, Pure Culture. Stained with Methylene Blue. (4 × eyepiece and 1/12 oil immersion objective.)

Of the various organisms that infect man, staphylococci are among the most frequent offenders, and the vast majority of such infections are caused by one or other of the following: staphylococcus albus, staphylococcus aureus and staphylococcus citreus. Practically, these are alike in all respects, except pigment production. Staphylococcus albus produces a white pigment; aureus, a pigment that varies from light golden yellow to deep brownish-yellow; citreus, lemon or greenish-yellow pigment. Chromogenesis appears when colonies develop on media in an aerobic atmosphere, under anaerobic conditions pigment is not produced. Old cultures tend to lose their chromogenic power.

Morphology.—Staphylococci are spherical, unicellular organisms, from 0.5 to 1.0 μ in diameter. The characteristic of their arrangement is irregularity. They occur in masses, some of which resemble bunches of grapes; irregularly arranged groups of three, four and five are numerous; fragmentation of these irregular masses results in accidental arrangement of a few in pairs or short chains as well as singly.

Motility.—In hanging-drop preparations Brownian movement is observed, but not true motility; staphylococci are non-motile.

Staining.—Staphylococci stain with all the usual anilin dyes and are Gram positive.

Culture.—Staphylococci are aerobic and facultative anaerobic. They grow at any temperature between 10°C. and 40°C., most luxuriantly at or near body temperature, 37°C. All the ordinary media are suitable for cultivating staphylococci. Media that are slightly alkaline are most favorable, but they grow well if the reaction is neutral or slightly acid.

Blood Serum.—On coagulated blood serum growth is rapid, at first transparent; then, the color becomes rather white, and within 24 to 48 hours chromogenesis appears. The colonies are round, elevated, smooth-edged and have a moist, glistening appearance. They vary in size, some smaller and a few larger than a pin head, in several days they coalesce and, after a time, slight liquefaction of the media is produced by some strains.

Agar.—Growth on agar slants in tubes appears the same as on serum, but liquefaction of agar never occurs. On agar plates colonies appear as already described; they tend to remain discrete; coalescence does not usually occur.

Gelatin.—Surface cultures on plates and slant tubes, incubated at room temperature 20°C. to 25°C.) show colonies within 24 to 48 hours. After several days the media adjacent to the colonies liquefies so that each colony is surrounded by a basin-like zone of liquid gelatin. As time goes on, liquefaction progresses, zones of liquefaction merge and finally the entire media becomes fluid.

Gelatin Stab Cultures.—First show granular growth along the stab; several days later liquefaction begins. It starts at the surface of the stab; as it progresses it becomes funnel-shaped, the greatest area of liquefaction being at the surface, the track gradually narrowing toward the bottom. A cloudy sediment, white or yellowish, forms in the liquefied gelatin.*

Bouillon.—Becomes cloudy in 12 to 24 hours; then, a white precipitate falls to the bottom, the media continuing cloudy. After several days the sediment becomes yellowish if staphylococcus aureus or citreus is present.

Milk is acidulated and coagulated.

Potato is the most favorable medium for pigment production. An abundant

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* Different strains of staphylococci vary in their effect on gelatin nitrates and carbohydrates. Some liquefy gelatin more slowly than others, some do not liquefy it at all. Some act on all the carbohydrates mentioned and others on but one, two or three of them. Some reduce nitrates and others do not.

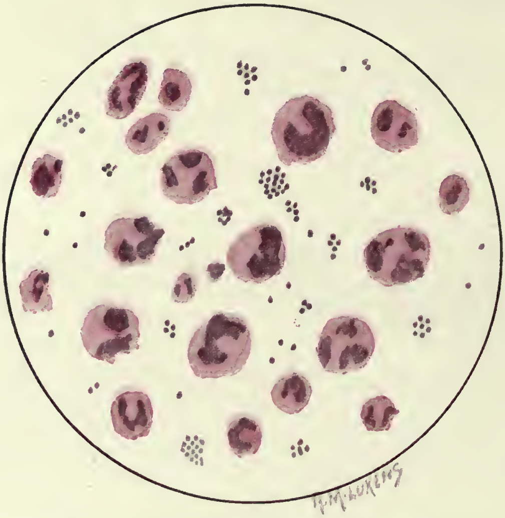

Fig. 11.—Spread from Pus Showing Staphylococci. Stained by Gram's Method
(4 × eyepiece and 1/12 oil immersion.)

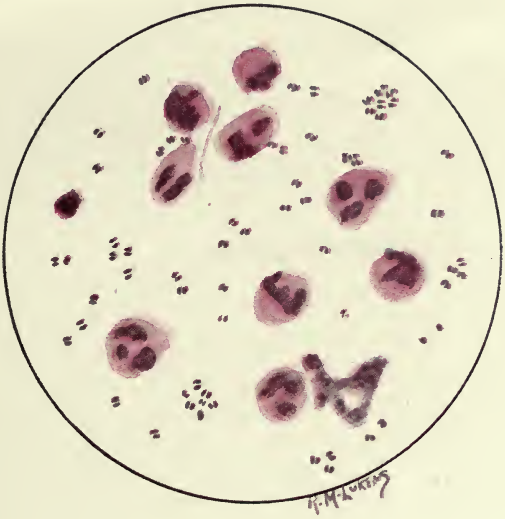

FIG. 12.—STAPHYLOCOCCI IN SPREAD FROM A CASE OF NON-SPECIFIC URETHRITIS. STAINED BY GRAM'S METHOD.

(4 × eyepiece and 1/12 oil immersion.)

growth covers the surface in several days and is white, golden or lemon-colored, according to the staphylococcus present.

Indol is not produced in 48 to 72 hours.

Media Containing dextrose, lactose, saccharose, maltose, mannite or glycerin may not be acidulated, usually they are, but gas is not formed.

Spore Formation.—Staphylococci do not form spores.

Resistance.—Staphylococci remain alive on culture media for months. Deprived of moisture they remain for several weeks or months. Repeated freezing does not kill them. When surrounded by or contained in albuminous matter they are especially resistant. Marked variations in resistance to chemical and thermal germicides are shown by different cultures; 1:1000 solutions of bichloride of mercury kills them in 10 minutes; 1:100 carbolic acid kills in from ½ to 2 hours. In a moist state they are usually killed by an exposure of ½-hour to 60°C.; some strains resist 80°C. for 1 hour. Boiling kills them almost instantly. In a dry state, 90°C. to 100°C. for ½ hour is required to kill them.

Toxin.—Staphylococci produce an intracellular toxin destructive to both red and white blood cells.

Agglutinins.—Specific for the staphylococcus have been produced experimentally, but cannot be regularly detected in patients infected with staphylococci.

PATHOGENESIS

Staphylococci vary in virulence, some are very virulent and others are incapable of producing disease. The majority of infections following wounds and operations and most of the suppurative processes, boils and abscesses are caused by staphylococci, usually staphylococcus aureus. No portion of the body is invulnerable to them. They are sometimes the offending organisms in rhinitis, otitis, coryza, pharyngitis, bronchitis, pneumonia, pleurisy, endocarditis, synovitis, enteritis, nephritis, cystitis, urethritis and meningitis. Staphylococci infections may be localized or widespread. Entering a wound they may lodge in adjacent tissue, injure or destroy it, but progress no further, as in single abscess formation. They may enter through a wound, invade the lymph glands, travel through the lymph vessels and lodge in various tissues and organs producing inflammation or suppuration at each focus. Likewise, they may enter the blood stream and be disseminated throughout the body or lodge in one or several organs.

Lower animals seem less susceptible to staphylococcus infection than man, but they are not immune.

Cultures injected into guinea-pigs, rats, rabbits, cats or dogs act as they do in man. Of the animals mentioned rabbits are most susceptible to staphylococci.

DIAGNOSIS

Upon inflamed surfaces and in pus produced by staphylococci the organisms are abundant. A loopful of pus, or scrapings from an inflamed surface, obtained with a cotton swab, when smeared on a slide, dried and stained, will disclose the nature of a staphylococcus infection; it does not indicate which staphylococcus is the offender; to determine this cultures on agar or gelatin must be made and chromogenesis observed.

When infection of the blood is suspected a conspicuous vein is sought, usually at the elbow, the skin overlying the vein is asepticized, a sterile needle is thrust into the vein, and from 2 to 10 cc. of blood withdrawn with a sterile glass syringe. The blood is immediately ejected into a flask containing 200 cc. of sterile bouillon. The flask is shaken and then incubated at 37°C. for 1 or 2 days. If growth appears a loop full of the bouillon is smeared on a slide, stained and examined; subcultures on agar and gelatin may be made.

When obtaining material from an inflamed surface, pus, blood or urine to examine for staphylococci, the common presence of staphylococci upon the surface of the body must be remembered and scrupulous precaution observed to prevent contamination, otherwise misleading findings may be made.

Opsonic index and complement fixation tests have a limited value in the diagnosis of obscure staphylococcus infections, but are seldom employed.

SERUM AND VACCINE THERAPY

Sera have been prepared to combat staphylococcus infections, but their value is slight. Vaccines or bacterins fortify or elevate natural immunity and so modify, limit and curtail infections, in favorable cases.

CHAPTER VIII

STREPTOCOCCI

Streptococci occur in air, water and soil, occasionally in horse manure and cow manure, sometimes on the skin and in the mouth of healthy people.

Morphology.—Streptococci are spherical, unicellular organisms from 0.5 to 1.0μ in diameter. They are arranged in chains. These chains may be short or long, composed of three or four cocci, a dozen or more; they may be straight, curved or tangled, especially the longer chains. Streptococci found in blood, spinal fluid, and cultures from them often appear in pairs, but on further cultivation assume their regular arrangement in chains.

Streptococci are non-motile, stain readily with anilin stains and are Gram positive.

Growth.—Streptococci grow well on ordinary culture media, having a slightly acid, neutral or slightly alkaline reaction. The optimum temperature for growth is 37°C., but though slowly, growth takes place at any temperature between 15°C. and 44°C.

Agar.—Surface growth on agar appears in 18 to 24 hours. The colonies are convex, raised, grayish-white, opalescent and have lace-like, slightly corrugated edges. They are pin-point in size and usually do not coalesce nor enlarge as the culture ages; after several days they tend to die out.

Gelatin.—Surface growth on gelatin is the same as that on agar, with the exception that colonies are more distinctly white.

Stab cultures in gelatin develop slowly, small, round, opaque, white colonies appear along the stab in from 24 to 48 hours, and the culture dies when 5 or 6 days old. With the exception of the streptococcus fecalis and possibly a few pathogenic streptococci, they do not liquefy gelatine.

Blood Serum.—On coagulated blood serum growth is the same as on agar, except that it is usually more luxuriant.

Potato.—Growth apparent to the naked eye seldom, if ever, occurs on this medium.

Milk is acidulated; some streptococci coagulate it and others do not.

Bouillon.—Cultures incubated at 37°C. show a light, white sediment in 24 hours; at first it sticks to the sides of the tube, later it falls to the bottom, in most cases leaving the medium clear. Lactic acid is formed. In bouillon streptococci find most favorable conditions for development of long chains.

Both bouillon and agar containing blood serum or ascitic fluid favor a more luxuriant growth of streptococci than plain bouillon and agar.

Blood agar (2 parts agar mixed with 5 parts whole human blood). On this medium different strains of streptococci show slight differences in growth.

Schottmüller has employed this medium to differentiate streptococci into several groups:

Streptococcus pyogenes or erysipelatos, grayish colonies surrounded by a zone of hemolysis.

Streptococcus viridans, greenish colonies, very slight, if any, hemolysis.

Streptococcus mucosus, slimy colonies, no hemolysis.

Streptococci do not form spores and do not produce indol.

Powers of Resistance.—Streptococci are much less resistant to germicidal agents than staphylococci. Pathogenic forms lose their virulence by cultivation on culture media; cultures die out in the course of days or weeks; drying rapidly reduces virulence and kills in the course of weeks; at 120°C. in hot-air sterilizer they are destroyed in ½ hour; in a moist state exposure to 60°C..for from ½ to 1 hour kills; boiling is almost instantly destructive. Saprophytic forms are more resistant.

TOXIN

Streptococci possess a variable intracellular toxin; attempts to produce an antitoxin have been futile.

Agglutinins do not appear in the blood of those infected with streptococci. Experimentally, animals can be made to produce agglutinins, but agglutination tests are unsatisfactory in diagnosis and bacteriological classification. The same is true regarding amboceptors and complement fixation tests.

Antistreptococci sera have not given satisfactory therapeutic results. Bacterial vaccines are of value in combatting infection and increasing resistance to streptococci, in selected cases.

PATHOGENESIS

Streptococci, like staphylococci, are strikingly variable in virulence; some are non-pathogenic, others but feebly pathogenic and some are extremely virulent. Virulence not only varies in different strains of streptococci, but individuals show marked variations in pathogenicity at different times. Virulence is increased by passage through animals, especially rabbits and man. It is decreased by cultivation on artificial media and saprophytic existence. Organisms isolated from a person whom they killed lose their pathogenic power to a large degree by cultivation on culture media for a long time. If such an attenuated culture is passed through several rabbits its original virulence may be restored.

Streptococci may be associated with disease either as its exciting cause or as secondary invaders, complicating or aggravating a condition that started as a staphylococcus infection, tuberculosis or other infection.

Streptococcus infections, like staphylococcus infections, may be localized, the organisms confined to a circumscribed portion of the body, or they may be septicemic, the organisms pervading the blood-stream.

Any portion or tissue may be involved; as a rule, localized streptococcic lesions show a greater area of inflammation and edema in proportion to the

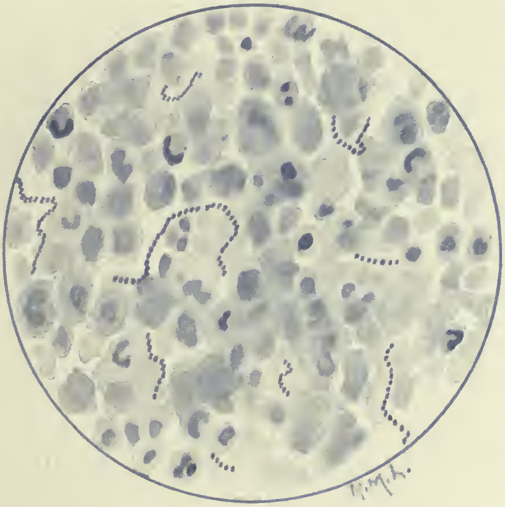

FIG. 13.—STREPTOCOCCUS IN A SMEAR FROM PUS. STAINED WITH METHYLENE BLUE. (4 × eyepiece and 1/12 oil immersion objective.)

amount of suppuration that occurs than do staphylococcic lesions, and the pus is more serous in character.

Some cases of broncho-penumonia, meningitis and angina are caused by streptococci. Streptococci are widely believed to be the specific cause of erysipelas; they are the common cause of puerperal septicemia, and though it is very doubtful, some believe they are the specific cause of scarlet fever, in which disease they are usually found present in the mouth and throat.*

Though not generally accepted at this time, one must bear in mind the teachings of Rosenow that streptococci may mutate and become pneumococci and *vice versa.* Rosenow strongly believes that the location of streptococci when they have entered the human body and the character of the disease which follows is determined by the particular strain of streptococcus infecting; different strains manifesting more or less constant selectivity or predilection to attack special organs or tissues, *e.g.*, one strain will affect the appendix, another the heart, another the stomach, etc.

Rosenow and others believe epidemic poliomyelitis is caused by streptococci.

DIAGNOSIS

Erysipelas and other inflammatory conditions, where pus is scant or absent, usually show some edema; by making a small incision in an appropriate place serous fluid may be obtained with a sterile capillary tube. From this several smears are made. These are stained and examined and will usually be found sufficient to establish the diagnosis in doubtful conditions caused by streptococci. However, organisms may not be found in the smears, or the morphology may be such as to make it impossible to say whether streptococci or pneumococci were observed; then it is desirable to make cultures. The fluid is obtained from the lesion with a sterile capillary tube and planted on several tubes of media—agar, blood agar, Loeffler's blood serum or other media. These tubes are incubated at 37°C. for 24 to 48 hours, and the growth examined both macroscopically and microscopically. Pus and sputum are examined for streptococci in the same way.

Where streptococcus bacteremia exists or is suspected, blood cultures must be made. The method of obtaining blood and inoculating media is the same as when making blood cultures for staphylococci, previously described.

Streptococci arranged in pairs rather than chains and sometimes encapsulated, so that they are indistinguishable from pneumococci under the microscope, are occasionally found, most often in examination of blood and sputum. Differentiation requires culture.

Inulin bouillon is fermented by pneumococci, not by streptococci; pneumococci form dry, blackish colonies on blood-agar without hemolysis; streptococci form whitish or greenish colonies with or without hemolysis; pneumococci grow in the sterile bouillon filtrate from streptococcus cultures, streptococci do not.

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* See "Further Experiments with a Streptococcus Isolated from Cases of Acute Rheumatism," Beattie, J. M., Journal of Pathology and Bacteriology, April, 1910.

"The Etiology of Erysipelas and Allied Infections," Panton and Adams, The Lancet, Oct. 9, 1909.

In doubtful cases differentiation of streptococci and pneumococci is difficult because intermediate forms not only vary from classic streptococci and pneumococci in morphology, but also in cultural characteristics, effects on rabbits, and behavior when mixed with agglutinins.

SERUM AND VACCINE THERAPY

A small percentage of cases of streptococci infection is greatly benefited by serum treatment, the condition being improved to a degree unattainable by other measures, but the majority is not influenced, and taken as a whole, serum treatment of streptococcus infections is still unsatisfactory.

The same is true of vaccine treatment of acute streptococcus infections, but in the treatment of chronic or recurrent streptococcus infections and the sequelæ of erysipelas, autogenous vaccines have a distinct value, and it is always advisable to employ them.

The extensive studies of streptococci and pneumococci executed in recent years by Rosenow and published in the Journal of the American Medical Association and other American journals of medicine, should be carefully read by advanced students. Among other things clearly disclosed is the fact that in some (possibly many) species of bacteria there are strains that will not grow in culture media under ordinary aerobic or anaerobic conditions; organisms that only grow in a certian oxygen concentration, the limits of which are narrow.

To insure growth of such organisms extra long test-tubes are filled with solid media nearly to the top. Either shake or stab cultures are made so as to distribute the plant from top to bottom of the tube. Under such conditions growth generally occurs in a zone ¼ to 1 inch in depth, the medium above and below appearing sterile. Such zones of growth may, with different organisms, develop at any level from near the bottom to near the top of the tube.

The proper oxygen supply or tension may be provided for such sensitive strains so as to induce growth by connecting the tube planted with streptococci to a tube planted with bacillus subtilis, with a small hose, in such a way that the cultures do not mix.

CHAPTER IX

PNEUMOCOCCUS

(Diplococcus lanceolatus)

The pneumococcus occurs in the nose and mouth of many healthy people, and in sputum from those who harbor the organism.

Morphology.—Pneumococci occur chiefly in pairs; they are not, as a rule, spherical, but lancet-shaped, one end oval the other slightly pointed; they are arranged with the long axis of each pair of cocci on the same pole, the oval ends in apposition, the pointed ends at the extremities of the long axis of each pair. Each pair of pneumococci is surrounded by a capsule when they are found in the body fluids; cultivated upon culture media, the capsule is nearly always lost. This capsule which surrounds each pair of cocci appears as a light halo surrounding the organisms stained in the ordinary way (with Loeffler's methylene blue, eosin, fuchsin or by Gram's); by special methods of staining the capsule may be tinted.

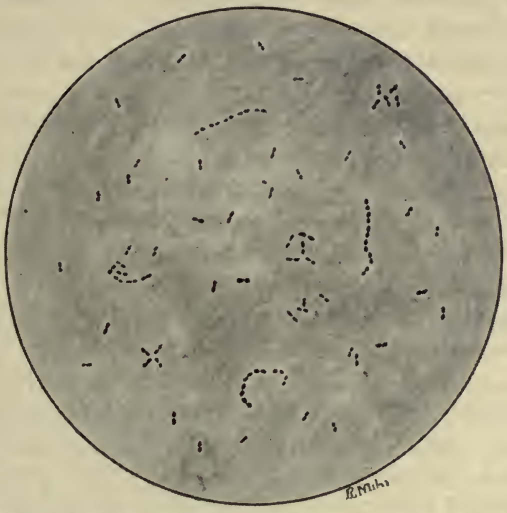

FIG. 14.—PNEUMOCOCCUS, FROM CULTURE. SHOWING IN PAIRS AND ALSO A FEW CHAINS; CAPSULES UNSTAINED. (4 × eyepiece and 1/12 oil immersion objective.)

Pneumococci may be found in chains, but their nature is usually discernible because there is usually a greater distance between each pair than between the elements of pairs, the diplococcic nature remaining apparent. Occasionally pneumococci are oval or almost spherical. Different strains vary in size, the average being slightly larger than staphylococci or streptococci. The pneumococcus **stains** with all the usual anilin dyes, capsule remaining unstained. It is **Gram** positive. There are various special methods of staining to tint both the cocci and surrounding capsule.

Growth.—The growth of pneumococci on culture media differs but slightly from streptococci; the range of temperature at which growth occurs is more restricted, from 25°C. to 41°C.; on solid media the colonies are more moist and transparent than streptococci and, as a rule, lack the elevated convex appearance of streptococci; under magnification intertwining chains and the lace-like edge of streptococci colonies are not observed. On blood-agar, colonies are dry, blackish, and do not cause hemolysis.

Litmus milk is more intensely acidified and more regularly coagulated by pneumococci. Pneumococci ferment inulin and acidify bouillon to a greater extent than streptococci. Otherwise the development on culture media is practically indistinguishable from streptococci.

Pneumococci do not form spores.

Resistance.—Surrounded by sputum or other viscid fluids pneumococci are more resistant to germicides than when brought into direct contact with the germicide; for this reason 20 to 50 per cent. alcohol is more destructive, because more penetrating than stronger, less diffusible substances, and will kill pneumococci in sputum in less than an hour. Pneumococci in masses of sputum dry out slowly and remain viable for several weeks; in droplets, especially if exposed to sunlight, they die in from a few hours to several days. They withstand low temperatures for a long time, freezing for weeks or months being required to kill them. Pneumococci are quickly destroyed by chemical germicides, in 5 per cent. carbolic or 0.1 per cent. bichloride of mercury solutions they are killed in several minutes. Ethylhydrocuperin, 1:500,000 solution inhibits growth, and 1:200,000 kills pneumococci *in vitro* in 3 hours at 75°C. Morgenroth and H. F. Moore have shown that sterilization of animals infected with the pneumococcus can be accomplished with this agent but the toxic dose is so close to the sterilizing dose that the danger of employing it precludes its therapeutic use. In a hot-air sterilizer pneumococci are killed in less than ½ hour at 120°C. In a moist state a temperature of from 55°C. to 60°C. kills in less than ½ hour.

Toxin.—Pneumococci produce an intracellular toxin; there is an antitoxin prepared that can neutralize it.

Agglutinins.—Can be produced experimentally but are not present in the serum of infected individuals in sufficient quantity to permit agglutination tests with the patient's serum for diagnosis.

Antipneumococci sera for therapeutic use were more unsatisfactory prior to the work of Cole than at present.

Complement fixation tests are not employed in diagnosis.

Bacterial vaccines do not confer immunity but are valuable adjuncts in the treatment of certain cases.

Pathogenesis.—Pneumococci have their virulence increased by passage through animals and lessened by cultivation or existence outside the animal body. It is believed that pneumococci which have been present in the mouth, nose or throat for aconsiderable time without producing any ill effect, can suddenly become virulent and cause pneumonia. Pneumonia caused by this organism ordinarily is not contagious, but at times there occur small epidemics that seem to indicate a high degree of contagiousness. Some epidemics of pneumonia caused by the pneumococcus are strikingly more fatal than others. None of these phenomena have been explained.

It is claimed by some that the pneumococcus is the offending organism in 90 per cent. of all cases of acute lobar pneumonia and in from 50 to 80 per cent. of all other forms of pneumonia. It is an established fact that the pneumococcus is the most common cause of pneumonia, but the figures given are probably too high.

In pneumonia the organism is present in the sputum and can frequently be obtained from the peripheral blood.

The pneumococcus is frequently the cause of otitis media, sometimes the cause of rhinitis and ozena, pleurisy, peritonitis, endocarditis, pericarditis and meningitis. If present in the lachrymal secretion at a time when trauma or operation causes a breach in the cornea or sclera, a serious infection often ensues.

When injected subcutaneously into rabbits a localized area of inflammation with much edema or suppuration at the point of inoculation may be the only effect, more commonly a fatal septicemia and death within 48 hours is the result. Under certain conditions, lobar pneumonia has been produced by spraying pneumococci into the lungs of rabbits and dogs.

Diagnosis.—In pneumonia microscopic examination of the sputum frequently is the only procedure required to establish the bacteriological diagnosis; blood culture is sometimes required and is carried out in detail as described for streptococci.

In localized lesions, such as pleurisy, the organism is found in the exudate. In meningitis the pneumococci are found in the spinal fluid.

In their efforts to produce a therapeutic serum, Dochez, Avery, Cole and others recently disclosed numerous important facts relative to pneumococci and pneumonia. They have shown that organisms identical in morphology, staining and cultural characteristics may be divided, by agglutination tests, into four groups or types that differ in distribution, pathogenicity and susceptibility to immune serum. Experiments have shown that among the various animals treated with these organisms horses produce the most effective therapeutic sera, and that the active principle of such sera is confined to the globulin portion.

Animals inoculated with **Type I** pneumococci yield serum which agglutinates **Type I** organisms but does not agglutinate any others; those inoculated with **Type II** pneumococci yield serum that agglutinates **Type II** organisms but does not agglutinate any others, animals inoculated with **Type III** pneumococci yield serum which cannot agglutinate **Type III** organisms until they have been divested of their capsule; and does not agglutinate any other organisms.

Type IV really is not a type—it is a heterogeneous group to which is relegated all pneumococci that are not agglutinated by **Type I** or **Type II** serums and lack the capsule, or fail, when denuded, to agglutinate with **Type III** serum.

Any **Type IV** pneumococcus when injected into an animal will produce a serum that will agglutinate cultures of the particular organism with which the animal was injected but will not agglutinate other **Type IV** pneumococci nor any other organisms.

The evidence so far accumulated shows that **Type I** serum administered to patients infected with **Type I** pneumococci and **Type II** serum administered to patients infected with **Type II** pneumococci exerts a more beneficial effect on the course and termination of the disease than any other therapeutic agent. An effective serum for the treatment of patients infected with **Type III** pneumococci has not been produced; the same is true of patients infected with **Type IV** organisms.

Type I serum has no effect on disease caused by any other organisms; **Type II** serum has no effect on disease caused by any other organisms; a polyvalent serum that might be employed with some benefit in any case, regardless of the type of pneumococcus offending, while most desirable, has not yet been produced.

Such observations as have been made during the last 2 years seem to show that the majority of cases of acute lobar pneumonia are caused by **Type I** or **Type II** pneumococci; that in different localities and years there are variations in the predominance of these, at one time **Type I** infections are more numerous than **Type II** and *vice versa;* that **Type IV** infections constitute about 25 per cent. of all cases; that **Type III** is the most virulent, **Type II** next in virulence and **Type IV** least; that the pneumococci found in the mouth or sputum of healthy and tuberculous persons, in practically all cases are **Type IV** organisms; and that the more virulent forms are present in the sputum of convalescent patients for a comparatively short time, as a rule.

It now seems desirable to discover to which of the four types the offending organism belongs in every pneumococcus infection and for this purpose a comparatively simple technique has been worked out.

IDENTIFICATION OF DIFFERENT TYPES OF PNEUMOCOCCI

Wash out patient's mouth with sterile water; have patient cough up some mucus from the throat or lungs; catch this sputum in a sterile container, put about 7 cc. of it in a centrifuge tube, add an equal quantity of sterile water, shake, centrifugalize at high speed for 15 minutes. Remove supernatant fluid, fill tube up again with sterile water, shake, centrifugalize at high speed for 15 minutes, discard supernatant fluid.

Draw the sediment into a sterile syringe and inject ½ cc. into the peritoneal cavity of a mouse; from 3 to 6 hours later chloroform the mouse, inject 10 cc. of normal salt solution into its peritoneal cavity (to collect the organisms that have multiplied) and withdraw it again. This fluid is centrifugalized just enough to free it of tissue cells.

Place five test-tubes in a rack and put an equal quantity of the washings from the mouse in each of the first three tubes—none in the last two. Put

Type I pneumococcus serum in the first and fourth tubes. Put **Type II** pneumococcus serum in the second and last tubes.

Serum is added in the proportion of 1 part serum to 29 parts of culture.

Add salt solution sufficient to make the quantity of fluid in all the tubes the same. Shake each tube to mix its contents and incubate at 37°C. for 2 hours or longer.

If agglutination occurs a whitish clump, like sputum, forms in the bottom of the tube, the supernatant fluid being clear. If agglutination does not occur there is no change in the appearance.

When, for any reason, it is impossible or undesirable to obtain the offending organism from the patient's sputum, a sterile glass syringe, fitted with a suitable needle is used to withdraw fluid from the affected lung; fluid so obtained is immediately transferred to a tube or flask containing bouillon and this is incubated at 37°C. for 12 to 18 hours; as growth appears the broth culture is employed the same as the washings from a mouse, in making agglutination test. Blood cultures, when procured, may be used likewise.

At the present time the identification of the form of pneumococcus producing pneumonia is being made by mixing equal quantities of patient's urine and serum. A positive reaction is indicated by turbidity within a few minutes after mixture. This test should be made within a few minutes after the urine has been withdrawn and after preliminary centrifugalization of the urine to make it perfectly clear.

SERUM THERAPY

Cole believes serum treatment should not be employed until agglutination tests have been made; that **Type I** serum should only be given to patients from whom **Type I** organisms have been isolated and that **Type II** serum should only be given to patients from whom **Type II** organisms have been isolated, that serum treatment should not, at present, be attempted when **Type III** or **Type IV** pneumococci are the offenders.

When serum treatment is indicated, the earlier in the disease it is employed the more beneficial its effect.

"When admitted, the patient was given 0.5 cc. of serum subcutaneously to discover if hypersensitiveness existed. As soon as the type of organism was determined, from 50 to 100 cc. of the serum, diluted one-half with salt solution, were injected intravenously.

"The condition of the patient served as a guide in the later treatment. Usually the serum was not administered oftener than every 12 hours. The patients treated received totals of from 190 to 460 cc. of serum, except one, who received a total of 700 cc. of serum."

CHAPTER X

MENINGOCOCCUS

(DIPLOCOCCUS INTRACELLULARIS MENINGITIS, WEICHSELBAUM)

The meningococcus occurs in the nose and perhaps the throat of some apparently healthy persons and occasionally in the nose of one suffering with meningitis.

Morphology.—The meningococcus is about the same size as staphylococci, but marked variations in size are observed in organisms obtained from the same source. They are arranged in pairs and are shaped like a coffee bean, oval with flat sides in apposition. The meningococcus stains with all the usual anilin dyes and is Gram negative. A peculiarity in staining sometimes observed is that different organisms in the same smear vary in staining, some being faintly stained and others deeply stained. Some observers have noted granular staining, one portion of a coccus staining more deeply than another.

The meningococcus is non-motile.

Growth.—The meningococcus is an obligate aerobic organism. Development on culture media occurs only at temperatures between 25°C. and 42°C.; best at 37°C. When a number of tubes of culture media are planted with meningococci obtained from the nares or spinal fluid only a few show growth. Meningococci which have led a saprophytic existence on culture media and have been transplanted several times possess a greater aptitude to grow on culture media; cultures from them nearly always show growth. Several cubic centimeters of pus or spinal fluid containing meningococci must be planted on media to obtain growth, even when microscopic examination discloses the presence of many organisms in the fluid.

Wherry and Oliver, also Cohen and Markle, state that partial oxygen tension cultures, made by connecting culture tubes with slant agar cultures of bacillus subtilis, with rubber hose, show abundant growth of meningococci and gonococci when aerobic cultures show scant and anaerobic cultures no growth.

Agar.—If growth occurs, transparent, pin-point-sized, round colonies appear in 24 hours, they become grayish and opaque in the center and remain discrete. After several days they die.

Glycerin agar shows the same character of growth as plain agar.

Blood-serum Agar, ascitic-fluid agar and blood-smeared agar are more favorable media for cultivation than plain agar and show a more luxuriant growth.

Loeffler's blood serum is the best solid medium for cultivation of the meningococcus. Pin-point grayish colonies appear in 24 hours, as on agar; the growth may become confluent.

Plain bouillon is not a favorable medium upon which to cultivate the meningococcus; when growth does occur it is scant and similar to that observed in bouillon which contains serum or ascitic fluid.

Serum Bouillon.—Slight cloudiness in 24 hours; after 36 to 48 hours a scant grayish-pellicle forms.

Milk is neither acidulated nor coagulated.

All cultures of the meningococcus die out in from 3 to 6 days, as a rule, but those which have been cultivated for a long time may survive longer than a week without transplanting.

Glucose and maltose are the only sugars fermented by this organism.

Resistance.—Drying kills the meningococcus in several hours, low temperatures and freezing kill in several days, 1:1000 bichloride and 1 per cent. phenol solutions kill in less than 2 minutes. In the hot-air sterilizer 100°C. for 15 minutes and in the moist state 60°C. for 10 to 15 minutes kills meningococci. Their resistance outside the body is slight.

Toxin.—The meningococcus produces an intracellular toxin of much virulence.

Agglutinins appear in the blood of persons having meningococcus meningitis and can be produced by injecting attenuated or killed cultures into animals. Complement fixation tests are employed to differentiate between meningococcus, gonococcus and micrococcus catarrhalis, but seldom in diagnosis.

Antimeningococcus serum is the most valuable agent employed in the treatment of meningococcus infections.

Pathogenesis.—In man the meningococcus causes meningitis, inflammation of the naso-pharynx and throat. Some observers have reported finding the organism in the blood of persons afflicted with meningitis. Conditions similar to those occurring in man can be produced by inoculation into monkeys. The organism is pathogenic for white mice, injected into the peritoneal cavity it produces a fatal peritonitis with exudate that contains many organisms. Other animals are immune or suffer from toxemia when inoculated.

Recent investigations have shown there are numerous strains of meningococci and para-meningococci which fail to agglutinate with serum from animals inoculated with the strains most commonly isolated from patients with meningitis—strains that are not acted upon by therapeutic sera on the market, hence the desirability of polyvalent sera.

Diagnosis.—In carriers the meningococcus is present on the mucosa of the naso-pharynx or throat. Suspected carriers are examined by passing a sterile cotton swab over these surfaces and then planting several tubes of blood serum and agar with the swab. Smears for microscopic examination are made from the swab.

When the disease is epidemic cases of naso-pharyngitis are examined as carriers, for it is believed that the meningococcus first assaults these membranes. Its activity may be confined to them or it may pass to the meninges and produce meningitis.

In cases of meningitis caused by the meningococcus the organism in nearly all cases may be found in the spinal fluid and exudate upon the membranes. The organisms are nearly all found within the pus cells, polynuclear leucocytes, some of these cells are full of cocci. These coffee bean-shaped, Gram negative, diplococci vary in size. Variations in size may be noted among those observed in a single pus cell; on an average they are somewhat larger than gonococci.

When spinal fluid is to be examined a strong needle about 6 inches long, that will not break if bent, is thrust into the spinal canal between the third and fourth lumbar vertebræ, about ¾ inch to one side of the median line, while the patient is lying on his side with thighs and head strongly flexed.

When the fluid begins to drop or flow from the needle, it is collected in a test-tube that will carry from 5 to 15 cc.

Strict aseptic precautions must be observed throughout, and after the needle has been withdrawn, the wound is painted with iodine and covered with a sterile dressing, the patient remaining reclined and at rest, for at least 8 hours.

SERUM THERAPY

A bacteriolytic serum is employed in the treatment of this disease, the usual method of administration being into the spinal canal.

CHAPTER XI

GONOCOCCUS

The gonococcus occurs in the genito-urinary tract of carriers and in the affected organs of infected persons. It is not found outside the human body.

Morphology.—The gonococcus occurs in pairs, the cocci arranged like coffee beans, ovoid with flat sides in apposition. The long diameter of the gonococcus is from 0.6 to 0.8 μ. It stains with all the anilin dyes and is Gram negative. The gonococcus does not form spores and is non-motile. Morphologically and by staining this organism is indistinguishable from the meningococcus.

Growth.—This organism cannot be satisfactorily cultivated on plain agar, gelatin or bouillon. Occasionally scant growth may be obtained in gelatin stabs (without liquefaction), on Loeffler's serum and on blood-smeared agar; but Wertheim's medium or some of its modifications serve best for the cultivation of the gonococcus.

To tubes containing 6 cc. of neutral glycerin agar, liquefied and cooled to 50°C., 4 cc. of sterile blood serum or ascitic fluid should be added. After mixing the serum and agar the tubes are slanted. Smears made on this medium show growth in 18 to 24 hours at 37°C. The colonies are round, pin-head size, whitish and tenacious. After from 3 days to a week they usually die out if not transplanted.

It is said that the addition of a piece of sterile rabbit testicle to culture medium favors the propagation of gonococci. While it is usually difficult to obtain a growth in tubes planted with gonococci directly removed from the human body, after the organisms have been successfully cultured and transplanted several times on media, they usually grow more regularly, more abundantly and less exacting as to the composition of the medium.

Gonococci will not grow in the presence of free moisture; there should be no water of condensation in tubes used for their cultivation.

C. C. Worden recommends the following medium for obtaining pure cultures of gonococci from material containing other organisms in addition to the gonococci, and states that it is a good medium for the cultivation of gonococci:

Sodium chloride	1.080 Gm.
Potassium chloride	0.045 Gm.
Calcium chloride	0.025 Gm.
Sodium bicarbonate	0.020 Gm.
Agar	0.250 Gm.
Nutrient broth	20 cc.
Distilled water	100 cc.

Filter through cotton into test-tube and sterilize once in autoclave.

Sugars are not fermented and indol is not formed.

Resistance.—The gonococcus is a delicate organism, in dried pus on linen and other articles usually it dies in several hours, occasionally it survives for several weeks. Freezing for a number of days eventually kills, but temperatures between 0°C. and 37.5°C. do not destroy it. Temperatures above 40°C. are injurious; in a moist state exposure to 60°C. for 10 or 15 minutes kills. Drying rapidly destroys gonococci; in a hot-air sterilizer they are killed at 100°C. in a few minutes.

Chemical germicides in very high dilutions are rapidly destructive.

The gonococcus is especially sensitive to salts of silver.

Toxin.—The gonococcus produces an intracellular toxin.

Agglutinins are not found in the serum of most infected patients; they may be produced by animal inoculation, but are not employed in diagnosis.

Attempts to produce antigonococcus sera of therapeutic value have been unsatisfactory.

Vaccines are of little or no value in acute infections; in subacute and chronic infections they sometimes give brilliant results; the chief indication for their administration is gonococcus arthritis.

Pathogenesis.—The gonococcus has a predilection for the mucous membranes of the urethra. It is most commonly found as the cause of urethritis. Gonorrhea is the most frequent of venereal diseases. The activity of this organism is not limited to the urethra in all cases; it often passes further, attacking the cord, testicles, bladder or kidneys, in the male, and the bladder, vagina, cervix, uterus, tubes or ovaries in the female. Occasionally infection is not localized in the genito-urinary tract, but passes to the blood stream and causes endocarditis, often malignant; the gonococcus may be deposited in the joints and cause arthritis; so-called gonorrheal rheumatism. Infection in practically all such cases is the result of sexual intercourse with persons harboring the organism.

An epidemic form of vaginitis caused by the gonococcus, not due to sexual intercourse, is observed from time to time among female children in the wards of hospitals. The mode of transmission in such cases is not known.

The gonococcus is sometimes conveyed to the eye by the fingers or linen soiled with urethral discharge or pus. It frequently gets into the eyes of infants during their passage through the vagina at birth, when the mother harbors the gonococcus. In the eye this organism produces a violent inflammation with profuse purulent exudate; not infrequently vision is impaired or totally destroyed.

The gonococcus may persist in the urethra or vagina long after manifestations of disease have ceased, when there is no pus, no discharge and when smears and cultures made from the urethra show no gonococci. Under these conditions the carrier, individual harboring the organism, may transmit infection during sexual intercourse.

The gonococcus does not infect any animal other than man, and does not exist outside the human body except under experimental conditions.

Diagnosis.—In acute infections of the genito-urinary organs and the eye,

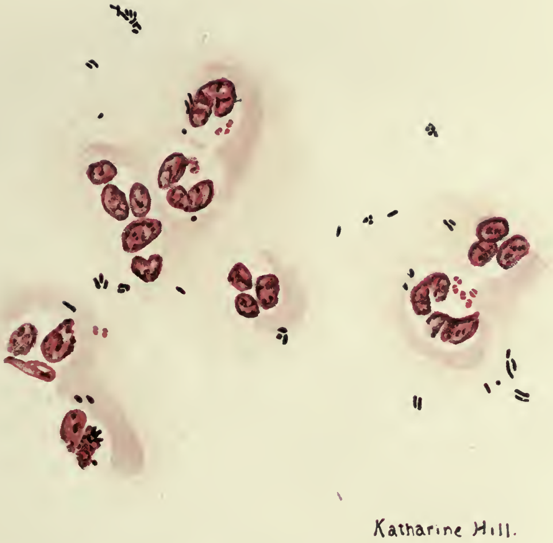
Katharine Hill.

Fig. 15.—Gonococci in Urethral Discharge. (Gram's Stain.) (*From Webster's "Diagnostic Methods."*)

gonococci are present in the purulent discharges. Smears made from such discharges and stained by Gram's method and counter stained with eosin or bismarck brown, show numerous pus cells, many of which contain Gram negative, coffee bean-shaped, diplococci; some pus cells contain only one or several pairs, others are crowded full of cocci; some cocci occur outside pus cells; these free cocci may show isolated pairs here and there or several pairs in a clump. Such findings, in the majority of cases, suffice to establish a diagnosis; culture is seldom resorted to for this purpose. If gonorrhea becomes chronic other organisms frequently invade the diseased tissue and may outnumber the gonococci to such an extent as to obscure them. When the disease becomes chronic without secondary infection, and in carriers, examination of discharge or secretion may not show the organisms. Under such circumstances the consumption of several glasses of beer, application of chemical irritants to the affected part, prostatic massage or an orgasm frequently will cause the appearance of gonococci in the urethral secretions.

Specific amboceptors are present and detectable to a sufficient degree, in cases of more than 6 weeks' duration, to make complement fixation tests valuable adjuncts in the detection of occult infections and hence in determining whether clinical cure is associated with bacteriological sterilization.

CHAPTER XII

MICROCOCCUS CATARRHALIS

The micrococcus catarrhalis is identical in appearance with the gonococcus and meningococcus and, like them, is Gram negative. It has only been found so far on the mucous membrane of the respiratory passages and in the sputum of some cases of pulmonary tuberculosis and lobar pneumonia patients. The organism has feeble pathogenic power and may cause catarrhal inflammations. It is of interest chiefly because it may be mistaken for the meningococcus. These organisms are easily distinguished by culture.

While it grows best at 37°C., the micrococcus catarrhalis will grow at any temperature down to 20°C.; the meningococcus will not grow below 25°. The micrococcus catarrhalis grows well on all the ordinary culture media, on gelatin, agar and blood serum incubated at 37°C., pin-head-sized, round, irregular-edged, white colonies form in 24 hours. In plain bouillon there is cloudiness with a fine white precipitate. Milk is not coagulated, sugars are not fermented.

CHAPTER XIII

MICROCOCCUS TETRAGENUS AND SARCINA

Micrococcus tetragenus is found occasionally in the mouth and sputum of healthy people. It is frequently present in the cavities and sputum of pulmonary tuberculosis and in the pus from abscesses and suppurative lesions of the mouth or naso-pharynx.

Morphology.—Micrococcus tetragenus is recognized by its arrangement in fours forming a square. These groups of four are usually surrounded by a capsule, but a capsule is not always visible. Each coccus is about the size of the staphylococcus. Micrococcus tetragenus is non-motile, stains with all the usual dyes and is Gram positive.

Growth.—All conditions favorable to growth of staphylococcus on culture media suffice for cultivation of micrococcus tetragenus.

On agar, potato and blood serum a luxuriant, thick, confluent, white growth appears in 24 to 48 hours at 37°C. Small, shiny white round colonies form on gelatin; it is not liquefied.

Milk is not coagulated.

When cultured on artificial media micrococcus tetragenus usually loses its capsule and very frequently loses its characteristic arrangement in fours so that the appearance of stained smears from cultures is often indistinguishable from staphylococci.

Hay infusion culture medium is said to favor the preservation of the arrangement in fours.

Resistance.—High and low temperatures and chemical germicides have the same effect on this organism as on staphylococci. Oral administration of sodium sulphite will cause the disappearance of sarcina in stomach contents.

Pathogenesis.—Micrococcus tetragenus is primarily a saprophyte. It usually enters diseased areas as a secondary invader, after some virulent organism has injured or destroyed tissue. It can cause abscess formations and pus either as a primary or secondary invader, usually the latter. At least one instance is on record of this organism having entered the blood-stream and causing septicemia. Its pathogenic properties and virulence are slight.

Injected into white mice a fatal septicemia may result. Injected into rabbits and other animals it causes an inflammation with or without pus formation, followed by rapid recovery in most cases.

Diagnosis.—The characteristic arrangement of micrococcus tetragenus suffices for its recognition in sputum, stomach contents, pus and other body tissues or fluids.

SARCINA

Sarcina are occasionally found in the mouth and sputum of healthy persons, more frequently when some chronic disease of the respiratory tract, as tuberculosis, exists. They are frequently present on foodstuffs exposed to air, dust and handling, and in certain foods, as eggs, undergoing putrefaction. Some forms are commonly present in air and soil.

Of the five varieties sarcina may be divided into according to the color of their growth on culture media:

Sarcina pulmonum (colorless).
Sarcina alba, and cervina (white).
Sarcina lutea, flava, and lactis (yellow).
Sarcina erythromyxa, (red).
Sarcina aurantiaca (orange).

None are pathogenic for man.

Morphology.—Sarcina are non-motile cocci about the size of staphylococci. They multiply by cell division in three directions and cocci remain united after fission, so they are arranged in cubes each surface of which presents four cocci or multiples of four. The appearance of these packets is often suggestive of a bale of cotton.

Staining.—Sarcina stain readily with all the common dyes and are Gram positive.

Growth appears, under aerobic conditions, at 37°C. in 24 to 72 hours on all the ordinary media.

Most strains do not coagulate milk; a few show acid but not gas production in glucose media, usually sugars are not acted on.

CHAPTER XIV

BACILLUS OF INFLUENZA, KOCH-WEEK'S BACILLUS, AND BORDET-GENGOU BACILLUS

The bacillus of influenza occurs in the mouth or throat of some healthy persons, in the saliva and sputum of influenza patients and in the droplets of mucus expelled with the breath. Outside the body, when dried, it dies in a few hours.

Morphology.—The influenza bacillus is very small, 0.3 to 1.5 μ.

Though usually rod-shaped, occasionally they resemble pneumococci, except that no capsule is possessed by the influenza bacillus.

Arrangement is irregular.

Bacillus influenza is non-motile and Gram negative.

Growth.—The bacillus of influenza or Pfeiffer's bacillus is an obligate aerobic organism that grows at temperatures between 26°C. and 42°C., best at 37°C. It requires the presence of hemoglobin for cultivation, does not grow on plain bouillon, agar or gelatin, poorly on blood-serum bouillon or serum-hemoglobin bouillon. Blood-smeared agar is the best medium for its growth. On this medium at 37°C., discrete colonies develop in 18 to 24 hours. Many of them are only visible when examined microscopically, some are pin-point size. They are round, dewdrop-like, do not coalesce and cultures die out in about a week unless transplanted. Growth is enhanced by cultivation in symbiosis with staphylococcus aureus and colonies pin-point to pin-head in size develop. Spores are not formed.

Resistance.—The bacillus of influenza is very perishable outside the body. In sputum it may survive for several weeks, but usually it dies in several days. Drying kills it in a day or two, 100°C. in hot-air sterilizer kills it in less than ½ hour. Moist heat at 50°C. kills in less than ½ hour. Chemical germicides in very weak solutions rapidly destroy it.

Toxin.—The influenza bacillus produces an intracellular toxin.

Agglutinins are not found in the blood of infected patients and experimentally, agglutinin production is irregular, not constant, and never very strong.

Complement fixation tests are not employed in the identification of this organism.

Pathogenesis.—The influenza bacillus is the exciting cause of some cases of coryza and influenza, occasionally it is the cause of pneumonia and meningitis. Bacillus of influenza infections occur sporadically, in epidemics and pandemics. In some cases the organism is present in the blood stream.

Guinea-pigs are immune to this organism. Rabbits and white mice die of septicemia following massive intraperitoneal inoculations. The pathogenicity of the influenza bacillus appears to be exalted by symbiotic development with staphylococcus aureus, pneumococcus and streptococci.

Diagnosis.—Whether the infection is localized in the upper air passages, or other parts are involved, the organism can usually be found in the nasal secretions and on the nasal mucosa of infected persons. It is present in the sputum when the bacillus of influenza produces pneumonia and in the spinal fluid when the infection affects the meninges. Smears stained by Gram's method and culture on blood-smeared agar usually suffice to establish the diagnosis. In doubtful cases inoculation of rabbits and guinea-pigs is of value.

When, as often occurs, the staphylococcus, pneumococcus or streptococcus is present in nasal secretion, sputum or spinal fluid, together with the influenza bacillus, they suggest a more serious condition than uncomplicated influenzal infection.

KOCH-WEEK'S BACILLUS

Koch-Week's bacillus, sometimes called the bacillus of acute contagious conjunctivitis, found on the conjunctiva and in the lachrymal discharge in cases of acute contagious conjunctivitis, is indistinguishable from the influenza bacillus and is considered to be the same by some authorities.

BORDET-GENGOU BACILLUS

Bordet-Gengou bacillus occurs in pure culture in the bronchial mucus and sputum of some cases of whooping-cough during the early days of the disease; later in the disease other organisms may also be present and make its recognition or isolation more difficult. When present in pure culture the bronchial secretion shows very many Bordet-Gengou bacilli, swarms of them.

Morphology.—It is a small bacillus, almost as small as the bacillus of influenza; it is oval, sometimes appearing like a coccus; it stains deepest at each end, and large numbers, close together, irregularly arranged, are found in smears from sputum.

The Bordet-Gengou bacillus stains with the usual anilin dyes and is Gram negative.

Growth.—The Bordet-Gengou bacillus does not grow on plain agar, gelatin or bouillon. Cultures direct from bronchial secretion do not develop so well as subcultures, those which have been cultivated and transplanted several times.

On Loeffler's blood serum, 1 per cent. glycerin-potato agar plus 100 per cent. blood serum, serum agar and ascitic fluid agar, growth occurs under aerobic conditions at 37°C. Glycerin-potato agar serum medium is the best for first cultures from the throat; subcultures do well on any of the media mentioned.

Microscopic growth develops on glycerin-potato agar serum in about 48 hours. Subcultures show a faint whitish growth which is said to become more abundant after a day or two.

Diagnosis is based upon a microscopic examination of bronchial secretion or sputum.

Vaccine therapy has been lauded by some who have employed it but is not in general use.

CHAPTER XV

BACILLUS OF MORAX AND AXENFELD

This organism causes a subacute or chronic catarrhal conjunctivitis. It is found in the exudate or pus of infected cases. Bacillus of Morax and Axenfeld is a diplobacillus, arranged in pairs, end to end. It is a large bacillus 2 to 3 μ long, 0.4 μ wide, has round ends, stains with the usual anilin dyes and is Gram negative. It does not form spores and is easily destroyed by heat.

Diagnosis is based upon microscopic examination of pus or exudate from suspected cases.

CHAPTER XVI

PNEUMOBACILLUS OR BACILLUS OF FRIEDLÄNDER

BACILLUS MUCOSUS CAPSULATUS

Bacillus Friedländer is widely distributed in nature, it occurs in air, water, soil, dust, milk, feces, saliva and sputum; in the mouth and sputum of some healthy people and in tissue attacked by this organism.

Morphology.—The pneumobacillus is about 1 to 4 μ long and 0.5 to 1.0 μ broad, has rounded ends and is surrounded by a distinct capsule. This capsule is apparent when organisms removed from tissue are observed; after cultivation on media the capsule may disappear. Pneumobacilli are arranged singly, in pairs and in chains. When in chains the capsules often merge as though one capsule enclosed the chain. This bacillus is not motile. It stains with all the usual stains and is Gram negative. Special stains are required to tint the capsule. In preparations stained by any of the ordinary methods the unstained capsule appears as a light, colorless zone surrounding the pigmented bacillus.

Growth.—The pneumobacillus grows well on all the ordinary media at temperatures between 15°C. and 40°C. Slightly acid, neutral or faintly alkaline media will do, but a slightly acid reaction is most favorable. Pneumobacillus is an aerobic and facultative anaerobic organism. Bouillon shows growth in 24 to 36 hours; it is clouded, a whitish pellicle forms on the surface and later a stringy whitish precipitate forms.

Gelatin stabs incubated at room temperature show a whitish growth in 48 hours. It follows the line of the stab and gradually extends over the surface, giving a tack-shaped growth. A few small gas bubbles may appear along the stab; the gelatin is not liquefied.

Surface cultures on gelatin show round, raised, grayish-white colonies in 2 or 3 days.

Agar.—In 24 to 36 hours a confluent, moist, white, tenacious growth covers the surface. On serum agar and Loeffler's medium growth is the same as on agar.

Milk is usually coagulated, rapidly by some strains and slowly by others; occasionally pneumobacilli are encountered, which do not coagulate milk, or at least fail until subcultured.

Indol is not formed. Some pneumobacilli ferment all the sugars; another group ferments all the sugars except lactose, and a third group ferments all sugars except saccharose.

The pneumobacillus does not form spores.

Resistance.—The pneumobacillus resists drying for a long time; an exposure for 1 hour in a hot-air sterilizer at 100°C. kills; in a moist state 60° to 70°C. for ½ hour kills. When not surrounded by sputum or albuminous matter carbolic acid (1 per cent. to 5 per cent. sol.) and 1:1000 bichloride solution kill in a few minutes.

Toxin.—Filtrates of bouillon cultures are toxic.

Agglutinins are not found in the blood of infected patients, but may be produced by inoculation of animals with killed cultures; results are uncertain and when an agglutinin is obtained it is specific for the particular strain of pneumobacillus used to produce it, it does not agglutinate other pneumobacilli, consequently agglutination tests are not of value in diagnosis. The same is true of complement fixation tests.

Friedländer's bacillus has been found the causative organism in pneumonia, septicemia, pleurisy, pericarditis, peritonitis, meningitis, stomatitis, tonsillitis and parotiditis. It can produce suppuration and abscess formation in many parts of the body but it is seldom the cause of pneumonia and more rarely the cause of septicemia, inflammation of endothelial tissues and suppuration. As a secondary invader it may be found in the discharge from chronic suppurating lesions, as gleet and otitis.

Rabbits are somewhat resistant or immune to the pneumobacillus; guinea-pigs and mice are susceptible when inoculated with virulent organisms, abscess formation at the point of inoculation, septicemia and death in several days follow.

Different strains of Friedländer's bacillus show marked variations in virulence; some are devoid of pathogenicity. For this reason and on account of variations in their effect on carbohydrates, some observers hold that bacillus Friedländer is the type of a group of closely allied organisms, some of which are pathogenic and some non-pathogenic. Among these closely allied bacilli, held by some to be typical Friedländer's and by others distinct entities, may be mentioned Bacillus of Rhinoscleroma, which is the exciting cause of some cases of chronic nasal catarrh, Bacillus Ozenæ, which is the exciting cause of fetid nasal catarrh, and Bacillus Lactis Aerogenes, found in air, dust, soil, water and especially in milk.

Diagnosis.—When the pneumobacillus is the cause of mucous membrane inflammations it is found in the surface exudate, it is present in the sputum when the exciting cause of pneumonia, in the exudate of pleurisy, pericarditis and peritonitis and in the pus of suppurating lesions. In the majority of cases diagnosis can be made by examination of smears stained by Gram's method and counterstained; occasionally culture for differentiation is necessary.

CHAPTER XVII

DIPHTHERIA BACILLUS

(Klebs-Loeffler Bacillus)

The diphtheria bacillus is found in the air, and dust and on the furniture and drapings of rooms inhabited by diphtheria patients and diphtheria carriers, on the clothing and utensils of diphtheria patients and carriers and perhaps on dogs, cats and cows associated with them. The organism sometimes finds its way into milk, probably from carriers and lives there virulent for a sufficient time to cause infection of those who consume the milk.

Morphology.—The diphtheria bacillus shows marked variations in size and form; the smears taken from the nose or throat and from 16 to 20-hour-old blood-serum cultures they usually appear as small bacilli, 2 to 3 μ long, 0.3 to 0.5 μ broad, many are straight or curved, some are club-shaped, a few triangular and some so small as to be coccoid.

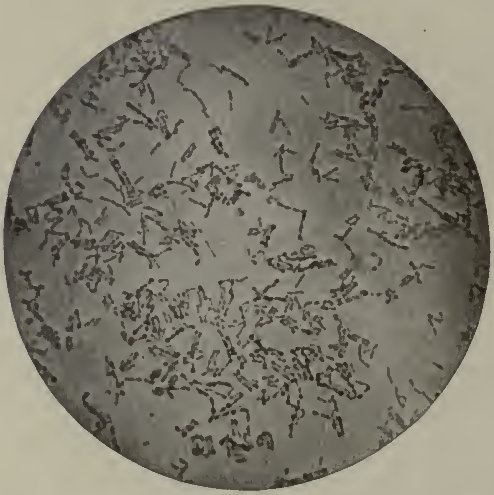

FIG. 16.—BACILLUS OF DIPHTHERIA. ×1000. (*MacNeal.*)

Smears made from cultures 24 to 72 hours old, or older, show involution forms, organisms more often club-shaped, balloon-shaped or irregular, than rod-shaped. Uniform staining is the exception, not the rule, especially among involution forms. The corynebacteria, of which the diphtheria bacillus is a type, when stained with the ordinary anilin stains, especially Loeffler's methylene blue, stain deeply at each end and faintly in the middle, or stain deeply at the extremities and in the center, giving a barred or granular appearance. When stained by Neisser's method—acid methylene blue, 10 to 15 minutes, washed in water and Bismarck brown applied for 1 or 2 minutes, the bars and granules appear deep blue, intervening portions light brown. The diphtheria bacillus is Gram positive. It is not motile.

There are so many variations in the morphology of true diphtheria bacilli and so many similar pseudodiphtheria and non-pathogenic bacilli that it is important to know them. For this reason and to assist in the differentiation between pathogenic and non-pathogenic forms Westbrook has described a classification which has been accepted by many health department laboratories and bacteriologists. It may be summarized as follows:

Type A.—*Decanter or Indian-club shaped*, showing one, two or more round granules, arranged together, or more frequently distributed; situated at one or both poles, or throughout the length of the organism.
1 μ to 2 μ thick
3 μ to 6 μ long

Type A^1.—Same shape and size as **A** but presenting bars in place of granules.

Type A^2.—Same size and shape, solid uniform staining.

Type B.—*Long slim straight or curved rods*, one, two or more round granules arranged together, or more frequently distributed; situated at one or both poles or throughout the length of the organism.
0.5 μ thick
3 μ to 7 μ long

Type B^1.—Same size and shape as **B** but presenting bars in place of granules.

Type B^2.—Same size and shape, solid uniform staining.

Type C.—*Straight or curved rods, frequently slightly swollen at one or both ends*, one or two round polar granules, and occasionally a third granule near the middle.
0.5 μ to 1.0 μ thick
3 μ to 6 μ long

Type C^1.—Same size and shape as **C** but presenting bars in place of granules, five or more bars may be observed.

Type C^2.—Same size and shape, uniform staining.

Type D.—*Straight or curved rods*, two round granules situated one at each pole.
0.75 μ to 1.00 μ thick
2μ to 3 μ long

Type D^1.—*Straight or slightly curved rod and carrot-shaped*, showing two, three, four or occasionally more bars.
0.5 μ to 1.0 μ thick
2 μ to 3 μ long

Type D^2.—*Isosceles triangle-shaped*, usually in pairs with bases in apposition solid and uniform staining.
0.75 μ to 1.00 μ thick
1.0 μ to 2.5 μ long

Type E.—*Straight or curved rods and ovoids*, two round granules situated one at each end.
0.50 μ to 0.75 μ thick
1 μ to 2 μ long

Type E^1.—Same size and shape as **E** but presenting two, three or four bars in place of granules.

Type E^2.—*Same size, blunt cone-shaped*, usually in pairs with bases in apposition solid and uniform staining.

Type F.—*Straight rods*, one round polar granule and occasionally a central granule.
0.25 μ to 0.50 μ thick
1 μ to 2 μ long

Type F^2.—Same size, irregular rod-shaped, solid uniform staining.

Type G.—*Ovoids*, two small round granules situated one at each pole.
0.50 μ to 0.75 μ thick
1.0 μ to 1.5 μ long

Type G^2.—Same shape and size as **G,** solid uniform staining. When stained with Loeffler's methylene blue, granular and barred forms of diphtheria bacilli are occasionally polychromatic, the granules and bars having a reddish tint. The metachromatic appearance is given as a characteristic of Westbrook's types **A, C, D, E, F** and **G.**

Growth.—The diphtheria bacillus is aerobic and grows at temperatures between 20°C. and 42°C., best at 37°C. The most favorable media for its cultivation are veal broth and Loeffler's blood serum.

Bouillon (preferably veal infusion) incubated at 37°C. after 24 to 48 hours shows a thin white film on the surface, later the growth precipitates to the bottom leaving the fluid clear.

Loeffler's blood serum on this medium, incubated at 37°C. round, elevated pin-point, grayish-white colonies appear in 16 to 20 hours; they enlarge, some attaining the size of a pin head or larger, others coalesce, forming an irregular outlined grayish film in 24 to 48 hours.

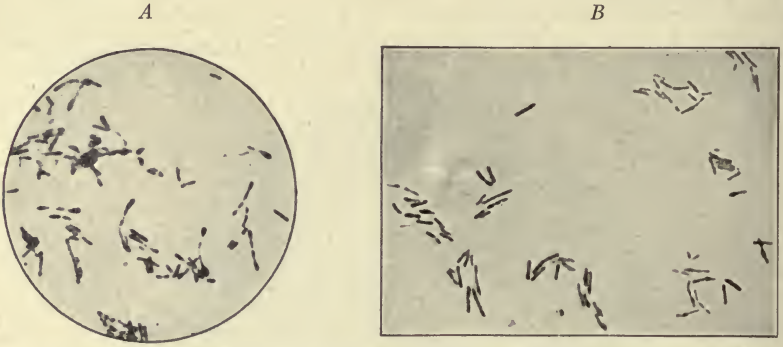

FIG. 17.—FORMS OF *B*, DIPTHERIÆ IN CULTURES ON LOFFLER'S SERUM. *A*, Characteristic clubbed and irregular shapes with irregular staining of the cell contents. × 1100. *B*, Irregular shapes with even staining. × 1000. (*After Park and Williams.*)

Some cultures have a moist appearance and some have a light yellow tint. Several days after inoculation the organisms show marked involution and the culture becomes dry and tends to die.

Agar.—On agar growth has the same appearance as on Loeffler's medium; it occurs more slowly, is less abundant, more distinctly white and does not die out as soon as on blood serum.

Gelatin.—A few pin-point white colonies develop along a stab; on the surface, occasionally, small round white colonies appear. Gelatin is not liquefied.

Milk.—Growth in this medium neither sours nor coagulates it.

Spores are not formed. Indol is not formed. Acid is formed from glycerin, glucose, galactose and maltose. Gas is not formed.

Resistance.—The diphtheria bacillus is easily destroyed by heat in a moist state, 60°C. for 15 minutes kills, but when dried, especially when enclosed in a false membrane, an exposure of 1 hour at 120°C. in a hot-air sterilizer is required to kill it. In water it lives a short time, and exposure to sunlight destroys it in several weeks. When dried and protected from sunlight it survives in dust, etc., for many weeks. Carbolic acid, 1 per cent. solution kills the diphtheria bacillus in less than 2 minutes; it is equally sensitive to other chemical germicides.

Toxin.—The diphtheria bacillus produces a powerful extracellular toxin. Different strains of the organism vary in this respect, some forming little and others much toxin. Alkaline veal infusion broth is the best medium in which to obtain toxin and it is best produced when incubation is maintained in a moist atmosphere between 35°C. and 37°C. For toxin production an abundant supply of oxygen (air) is required.

Diphtheria toxin is filterable, hence after the bacillus has produced toxin in bouillon, by filtering the culture through an unglazed porcelain tube the toxin is recovered in the filtrate free from bacteria. As small an amount as $\frac{1}{200}$ cc. of such a filtrate often contains sufficient toxin to kill a guinea-pig.

Stored in a cool dark place diphtheria toxin gradually deteriorates in the course of months; heating above 58°C. rapidly attenuates it, 100°C. destroys in a short time.

The diphtheria toxin is water-soluble and alcohol-insoluble.

By injecting subcutaneously varying amounts of toxin into different guinea-pigs of the same weight the lethal dose can be determined.

A subcutaneous injection of a very small fraction of the lethal dose of anti-toxin acts on some animals in such a way as to increase resistance in 5 to 10 days; a second injection of a quantity of toxin 50 to 100 per cent. greater than given at first will then further increase the animal's resistance and by continuing the treatment, giving subcutaneous injections of progressively larger doses of toxin at intervals of about a week until from 6 to 12 injections have been administered, a high degree of resistance or immunity is conferred upon the animal.

Blood serum obtained from an animal so immunized when mixed with diphtheria toxin neutralizes it, makes it inert, and deprives it of disease-producing power. Such serum is known as diphtheria antitoxin and is used in the treatment of diphtheria. Serum obtained from animals immunized against diphtheria toxin does not contain agglutinins or amboceptors.

Agglutination tests and complement fixation tests are not available in the diagnosis of diphtheria, nor to differentiate diphtheria bacilli from pseudo-diphtheria bacilli.

Pathogenesis.—The diphtheria bacillus has a selective affinity for the mucous membranes of man, especially the mucosa of the upper air passages. This organism may be found upon the mucous membrane of the throat, tonsils or nose of healthy people who have never had the disease; this is a common occurrence among those in contact with diphtheria patients and relatively rare among other healthy persons. Healthy persons who harbor the diphtheria bacillus, so called carriers, are dangerous, and may transmit the bacilli to others in whom the organisms promptly cause disease. Epidemics of diphtheria have been traced to such sources, especially among school children.

After a diphtheria patient has entirely recovered from the disease he may still harbor the bacillus for days, weeks or months.

In the vast majority of cases the diphtheria bacillus lodges upon the mucous membranes of the nose or throat, mostly on the tonsils, not infrequently on the larynx. If active it causes a false membrane to form on these surfaces. This membrane is at first dirty white or grayish, as it ages it becomes darker. When stripped off, the exposed mucous membrane is inflamed and may show bleeding points. Although the diphtheria bacilli are confined to the mucous and false membrane and do not penetrate underlying structures nor enter the blood- or lymph-streams, diphtheria is a disease with profound systemic toxemia. The toxin liberated by the bacilli upon the mucous membrane is absorbed and disseminated through the blood-stream.

Rare, isolated cases have been reported in which the diphtheria bacillus has attacked the ear, the intestine and the genito-urinary canal.

Occasionally the pus from chronic urethritis of long duration, when examined microscopically, shows, among other organisms, bacilli morphologically indistinguishable from diphtheria bacilli. Most of these are not diphtheria bacilli, some produce toxin in broth and apparently are true diphtheria bacilli. Whether diphtheria bacilli or not, they do not cause false membrane formation and do not cause toxemia; they are active in maintaining the urethritis, as evidenced by rapid recovery after they cease to exist in the urethra.

The diphtheria bacillus, injected subcutaneously, is virulent for horses, cattle, dogs, cats, rats, guinea-pigs, chickens and many other birds and animals. Cats are said to be subject to diphtheria.

Marked variations in virulence and toxin production are exhibited by different strains of diphtheria bacilli. The virulence and toxin production of any diphtheria bacillus depends, to a large extent, upon little understood conditions of environment. A diphtheria bacillus may exist in one person's throat for days or weeks without injury to the host and immediately cause disease when transferred to another. A diphtheria bacillus cultivated in bouillon may produce so little toxin that 10 cc. or more of the filtrate is required to kill a guinea-pig, but when the same organism is transplanted into a different bouillon, one more nearly adapted to its requirements, so much toxin will be produced that 0.01 cc. or less of the filtrate will kill a guinea-pig.

Some strains of diphtheria bacilli are more constant in their degree of virulence and toxin production than others and less susceptible to changes in environment.

Diagnosis.—When the diphtheria bacillus is present in the nose, throat or tonsils, a sterile cotton swab passed over the infected surface and then smeared on a glass slide may deposit bacteria on the slide that will be visible when stained and examined microscopically, or it may not. If after passing a sterile cotton swab over the infected area, the swab is then drawn across the surface of Loeffler's blood serum and the medium incubated 16 to 24 hours at 37°C., any bacteria introduced will multiply and form colonies from which slides may be prepared and stained for microscopic examination and the organism examined. It is desirable to establish the bacteriological diagnosis in suspected cases of diphtheria early as possible, and for this reason both culture and slides should be obtained from the patient.

Occasionally diphtheria bacilli will be found in the nose and not in the mouth or throat, hence slides and cultures should be made from both the nose and throat.

TECHNIQUE

Take two tubes of sterile Loeffler's blood-serum medium, two sticks or pieces of wire about 6 inches long having cotton wrapped around one end (when made such swabs are placed in test-tubes stoppered with cotton, and the tubes containing swabs are sterilized at 140°C. for 1 hour in hot-air sterilizer) and several clean glass slides.

Inspect the patient's mouth and throat; if grayish-white spots or areas of inflammation are observed rub the swab over these and immediately draw across the surface of the Loeffler's medium; then rub the swab on one or more glass slides and replace it in its original container. If no grayish spots or areas of inflammation are observed draw the swab over each tonsil and the pharynx.

Take the second swab and pass it into each nostril, then draw it across the surface of a sterile tube of Loeffler's medium and make slides from it; finally, replace this swab in its original container.

Place the blood-serum culture tubes in incubator at 37°C.; stain the slides for 5 minutes with Loeffler's methylene blue, wash them in water, dry and examine under the microscope. If diphtheria bacilli are observed the diagnosis is apparent.

After 16 to 24 hours remove cultures from incubator, note the macroscopic appearance of colonies that have developed; remove some of the growth with a sterile platinum loop and smear on slides, fix by gently heating slides until dry, stain with Loeffler's methylene blue and examine microscopically.

When slides and cultures from a true case of diphtheria are examined many diphtheria bacilli are usually found, occasionally they are few. They may be found in pure culture—no other organism appearing; as a rule, however, staphylococci, streptococci or pneumococci are found together with the diphtheria bacilli.

Usually virulent diphtheria bacilli, as observed in smears made directly from the nose and throat or from 16 to 20-hour-old blood-serum cultures, are slim, straight and curved rods showing granular or barred staining.

When such smears show, almost entirely, solid staining, short, stout rods, the organisms usually are not virulent, toxin-producing bacteria.

There are so many exceptions to these rules, however, that occasional error is inevitable when diagnosis is based on the appearance of bacteria, and under such conditions the welfare of patients and public is best served by making a diagnosis of diphtheria whenever smears or cultures obtained from a patient having sore throat, fever and signs of toxemia, show organisms having the morphology of either the diphtheria or pseudodiphtheria bacilli.

Shick Test.—One of the recent additions to diagnostic tests has disclosed much valuable information in regard to the prevalence of immunity and susceptibility of persons of various ages in civilized communities and promises to be of practical value in the future to determine whether or not any individual is immune or susceptible to diphtheria.

The test is based on the findings of von Behring and others that if one has as much as $\frac{1}{30}$ unit of antitoxin per cubic centimeter in his blood serum he is immune. An amount of toxin which requires the presence of $\frac{1}{30}$ unit antitoxin per cubic centimeter to neutralize it is injected into the skin. If it is neutralized inflammation does not occur. If not neutralized, in 24 to 48 hours, an area of erythema 1 to 2 centimeters in diameter appears with slight infiltration. Shick recommends the use of $\frac{1}{50}$ unit of toxin in 0.1 cc. sterile water.

The toxin should not be diluted until the test is to be made; the injection must be into and not beneath the skin, if properly injected a slight swelling and blanching occurs at the point of injection.

Strong or concentrated toxin should be employed so as to minimize the amount of protein from culture medium injected because this is thought to be responsible for the pseudoreactions at times observed.

Carriers.—Persons who harbor diphtheria bacilli in nose or throat without suffering any injury therefrom are known as carriers. Numerous investigations have shown that most patients continue to have diphtheria bacilli in their nose or throat for 1 to 2 weeks after the beginning of convalescence from diphtheria; from 0.5 to 5 per cent. continue to be carriers for weeks or months; and from 0.1 to 3 per cent. of persons, who, so far as known, never had diphtheria, harbor virulent bacilli at times or continuously. These carriers are most numerous among nurses and physicians attending diphtheria patients.

Pseudodiphtheria bacilli are occasionally or continually present in the nose or throat of from 10 to 80 per cent. of all people in numerous localities.

Obviously it is necessary to determine that a suspected or probable carrier is free of diphtheria bacilli before release from quarantine and that organisms obtained from a suspect be tested for virulence as well as examined microscopically.

Kolmer and Woody recommend the following virulence test of organisms isolated from convalescents and suspected carriers:

(*A*) 1. Obtain culture from patient on Loeffler's blood serum. 2. Transplant to 0.2 per cent. glucose broth +0.8 reaction. 3. Incubate broth 72 hours. 4. Inject, subcutaneously, an amount of the culture equal to ½ per cent. the test animal's weight—use 250 to 300 Gm. guinea-pigs.

(*B*) 1. Procure a good 24-hour-old culture from suspected patient on Loeffler's medium. 2. Wash off growth with 10 cc. of salt solution. 3. Inject 4 cc. of this salt solution suspension subcutaneously into a 250 to 300-Gm. guinea-pig.

The test animal is observed for 4 days and if inflammation at point of injection and toxemia occur the organism is considered virulent.

CHAPTER XVIII

PSEUDODIPHTHERIA BACILLI

There are a number of organisms, not pathogenic, which are similar to the diphtheria bacillus morphologically, in staining and growth on culture media. "They have been found in pus, milk, urine and infectious processes resembling the true diphtheria bacilli" (Rosenberger).

"Differentiation is never difficult" (Hiss and Zinsser). "Bacillus diphtheriæ forms acid from dextrin, not from saccharose; Bacillus xerosis from saccharose not from dextrin; Bacillus Hoffmanni does not form acid from either" (Hiss and Zinsser).

Hoffman's bacillus is found in the throat of healthy people, not infrequently, especially among school children in poor districts. Morphologically it is said to be shorter than the diphtheria bacillus and more uniform in shape. It is also claimed that upon staining Hoffman's bacillus does not show the irregular or band-like staining common to the diphtheria bacillus and when stained by Neisser's method the entire bacillus is brown, no blue staining granules visible.

Pseudodiphtheria bacilli have a very wide distribution. They are frequently found as secondary invaders in chronic suppurative processes exposed to air. They are occasionally discovered in soil and on various utensils. They have been regularly obtained from the infected tissues in Hodgkin's disease, and are believed by some to play a part in this disease. As to whether they constitute the specific factor of Hodgkin's disease is a mooted point; the preponderance of evidence being against it.

Growth.—On agar, blood serum, gelatin and bouillon the growth of Hoffman's bacillus is similar to bacillus diphtheria, but more luxuriant. In contrast to the diphtheria bacillus it does not acidulate media containing any of the sugars.

Pathogenesis.—Hoffman's bacillus does not produce extracellular toxin, does not kill guinea-pigs nor rabbits and is not pathogenic to man.

The xerosis bacillus is most often found upon the conjunctiva, both in health and when the eye is the seat of disease. In morphology and staining it resembles the diphtheria bacillus even more closely than Hoffman's bacillus.

Growth.—The xerosis bacillus may be cultivated on any of the media upon which the diphtheria bacillus grows; its growth is finer, less abundant but similar to that of the diphtheria bacillus. It forms acid on saccharose, but not on dextrin.

Pathogenesis.—The xerosis bacillus is not pathogenic for man, guinea-pigs nor rabbits. It does not form toxin.

Diagnosis.—With few if any exceptions diphtheria and pseudodiphtheria must be thought of and a differentiation made whenever an organism presumably a pseudodiphtheria bacillus is found associated with an infection of the nose or throat. Experienced clinical bacteriologists can usually do this by observing the morphology and staining of microscopic preparations together with the character of growth on Loeffler's blood-serum medium. It is one of the most difficult problems that confronts the beginner.

While there is much confusion and disagreement on the subject it seems most probable that we have to deal with organisms that naturally divide themselves into three groups:

First, true diphtheria bacilli which have temporarily lost some of their faculties.

Second, several distinct species—not diphtheria bacilli—neither pathogenic or toxin producers, including Hoffman's bacillus and the xerosis bacillus.

Third, a numerous heterogeneous group of non-pathogenic saprophytes more or less similar to and always distinguishable from typical diphtheria bacilli. This third group is almost ubiquitous.

CHAPTER XIX

THE BACILLUS AND SPIRILLUM OF VINCENT

Vincent's angina is an infection of the tonsils, mouth, pharynx or tongue caused by two organisms in symbiosis—the bacillus of Vincent, sometimes called fusiform bacillus, and the spirillum of Vincent.

The bacillus of Vincent, or bacilli indistinguishable from it, are occasionally found in the mouth, not associated with the spirillum of Vincent, when there is no disease.

The spirillum of Vincent, or spirilla indistinguishable from it, are occasionally found in the mouth, not associated with the bacillus of Vincent, when there is no disease.

Whenever both these organisms coexist in the mouth there are always obvious signs of disease; the evidence indicating that it is caused by this spirillum and bacillus and dependent upon their symbiotic relation.

Reported findings indicate that these organisms seldom, if ever, attack any part of the body other than the mouth and pharynx; that they remain localized in the superficial lesion and that their injurious effect is largely, probably entirely, limited thereto.

Morphology.—The bacillus of Vincent is fusiform, spindle-shaped, or, more often, shaped like a banana; it may be perfectly straight, but frequently is slightly curved. Typical organisms are thickest in the middle and taper to pointed ends. The bacillus of Vincent is about 5 to 10 μ long and 0.6 μ wide in the middle. It is not motile.

The spirillum of Vincent varies from 5 to 20 μ in length, is slender in proportion and shows irregular curves—irregular as to number and depth—sometimes they are very distinct and again very faint.

Staining.—Both the bacillus and spirillum of Vincent stain with the usual anilin stains, best with carbol fuchsin, and are Gram negative.

Growth.—The bacillus and spirillum of Vincent cannot be cultivated except under anaerobic conditions at 37°C. Growth appears on serum-agar and Loeffler's blood serum in 2 or 3 days as pin-point, round, grayish colonies. These colonies enlarge and may attain a diameter of 2 millimeters and may show a yellow tint.

Many tubes of serum agar and other media, regardless of how planted and incubated, fail to show growth. Attempt to obtain cultures are frequently frustrated by overgrowth of other organisms present in the material taken from lesions.

Pathogenesis.—The name angina was given to the infection caused by the spirillum and bacillus of Vincent because the first cases studied presented the superficial clinical appearance of diphtheria. Subsequent observations have proved this a misnomer.

At least three distinct clinical forms are observed:

First, acute onset, beginning as stomatitis, pharyngitis or unilateral tonsillitis, the inflamed area covered in several days by a pseudomembrane and presenting the appearance of diphtheria—after the pseudomembrane is cast off, the subsequent course may be as in diphtheria or slight superficial ulceration or deep ulceration may occur and persist for several days or weeks—those cases which at first are clinically indistinguishable from diphtheria are about 50 per per cent. of the whole.

Second, ulceration, most frequently involving one tonsil, rarely occurring on tongue, occasionally on gum, buccal mucosa or pharynx— ulcer developing as do syphilitic ulcers, having the punched-out appearance and other physical characteristics of a chancre—frequently unaffected by any form of treatment and persisting for weeks or months—these cases which always suggest syphilis constitute about 40 per cent. of the whole.

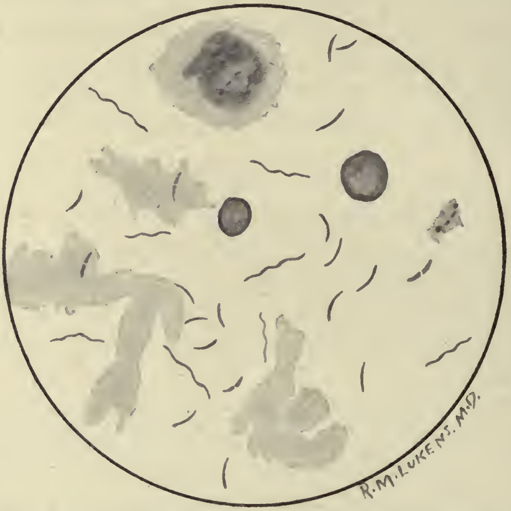

Fig. 18.—Spread from a Case of Vincent's Angina. Showing Spirilla and Fusiform Bacilli. Stained with Methylene Blue.
(4 × Eyepiece and 1/12 oil immersion objective.),

Third, insidious onset, of months or years duration and marked by the occurrence, most commonly on the gums, of one or more vesicles which develop and subside repeatedly—all of which contain bacilli and spirilla, occasionally these vesicles ulcerate—these cases form a very small per cent. of the whole.

Diagnosis.—In all cases of pharyngeal, tonsillar or buccal infection or ulcer a sterile cotton swab should be passed over the inflamed area and then rubbed on slides to be fixed, stained and examined microscopically.

At the same time a tube of Loeffler's medium should be inoculated and incubated aerobically at 37°C.

Both these procedures are advisable for the following reasons:

If the condition be diphtheria, slides examined microscopically, frequently fail to disclose diphtheria bacilli, while after incubation the cultures do reveal them.

If the condition be Vincent's angina, slides will always show fusiform bacilli and spiralla; aerobic cultures never do.

If, as sometimes occurs, a double infection exists, both Vincent's angina and diphtheria, it is well to recognize it, as such a condition is said to be more grave than diphtheria alone.

Some authorities state that slides made from mild cases of Vincent's angina show relatively smaller numbers than slides from severe cases. Usually the organisms are quite numerous. As a rule, several or more organisms other than the spirillum and bacillus of Vincent are found in slides made from cases of Vincent's angina—staphylococci and streptococci most frequently. At times the spirillum and bacillus of Vincent are found in practically pure culture.

CHAPTER XX

THE TUBERCLE BACILLUS

The tubercle bacillus occurs in air, dust, soil and water as a result of contamination with feces, urine, sputum or other discharges from tuberculou animals. It is frequently present in the milk of tuberculous cows and in products made from it, such as butter and cheese. Muscle tissue is rarely involved, but tubercle bacilli are numerous in the glands, lungs and viscera of diseased animals. Through contamination by dust and dirt and handling by tuberculous individuals, various articles of clothing and food are polluted with tubercle bacilli.

It is believed that bed-bugs inhabiting the bed of a tuberculous patient acquire tubercle bacilli, harbor them for long periods, and, when opportunity occurs, bite people and in so doing, infect them.

This organism has a wide distribution in every climate and may enter man through the respiratory tract, by inhalation, through the gastro-intestinal tract, by food, especially raw milk, possibly through the genital organs, by sexual intercourse, and through infection of wounds. In the great majority of cases, tuberculosis in man follows infection through the respiratory tract, of the rest most infections in man are by way of the intestinal tract.

Morphology.—Tubercle bacilli usually appear as small, straight or curved, solid-staining or beaded rods, 1.5 to 4.0 μ long and 0.3 to 0.5 μ wide. They may be arranged singly or in irregular clumps. Organisms removed from old cultures are often larger than those found in tissue and frequently show branching. Beaded forms of tubercle bacilli may look like several small cocci in a row.

Staining.—The peculiar reaction of the tubercle bacillus to stains, acids and alcohol facilitate the recognition of this organism microscopically. With the exception of the leprosy bacillus it is probably the only organism which is both acid- and alcohol-fast. Presumably on account of its fatty capsule, the usual anilin stains penetrate it with difficulty or not at all. When stained with carbol fuchsin neither acid nor alcohol decolorizes it and other stains cannot tint it.

Of the many methods of staining for the tubercle bacillus the following two are probably the best:

1. Stain with carbol fuchsin for 15 minutes or longer.
2. Immerse in Pappenheim's solution for 15 minutes, or until the slide looks blue.
3. Wash in water.
4. Dry and examine.

1. Stain with carbol fuchsin for 15 minutes or longer.
2. Wash with 5 per cent. aqueous solution of nitric acid until bleached (*i. e.*, pale pink).

3. Apply absolute alcohol for 30 seconds.
4. Wash in water and apply methylene blue for 5 minutes.
5. Wash in water.
6. Dry and examine.

By either of these methods tubercle bacilli will be stained red and everything else blue.

Growth.—The tubercle bacillus (human and bovine types) is an aerobic organism which grows at temperatures between 30°C. and 42°C., best at 37°C. It will not grow on media other than blood serum unless blood, blood serum, tissue or glycerin are added. Development is slow, growth first appears after 12 or 14 days' incubation and continues to increase for 3 or 4 weeks.

Organisms transplanted directly from the human body or sputum to culture media often fail to grow. Isolation of tubercle bacilli from other organisms by plating is unsatisfactory. It is best to inoculate a healthy animal with the suspected material or material containing tubercle bacilli or tubercle bacilli and other organisms. After 3 or 4 weeks general tuberculosis develops and if the animal is opened under aseptic precautions and the contents of enlarged tubercular glands removed and planted on appropriate culture media, pure cultures can be obtained.

Glycerin Bouillon.—To obtain growth on this medium several large loopsful of an actively growing young culture must be obtained and floated on the surface of the glycerin bouillon. The glycerin bouillon may have 1 per cent. glucose added to it with advantage; it must be slightly alkaline and have a good supply of oxygen, obtained by placing the bouillon in a broad flask with a wide neck. After 2 to 3 weeks' incubation at 37°C. growth appears on the surface; at first a small, thin whitish scum, which rapidly spreads over the entire surface and continues to grow thicker for a month or more. The pellicle becomes wrinkled or granular as it ages, portions may fall to the bottom of the flask, and when the culture is old the growth may change from white to yellowish. The medium remains clear.

Glycerin-agar.—Growth appears in about 2 weeks; at first as dry, scaly white spots. These coalesce, forming a thick, dry, corrugated film, described as "bread-crumb" growth.

Serum-agar.—Growth is the same as on glycerin-agar except that this is a more favorable medium for initial cultivation after removal from a guinea-pig.

Coagulated Serum.—Growth is the same as on serum-agar.

The tubercle bacillus does not form spores.

Resistance.—Tubercle bacilli are most resistant when in tissue, least resistant when in culture media. Putrefaction of tissue containing them does not lessen their vitality nor virulence. To destroy tubercle bacilli in tissue, sputum, feces, etc., boiling for ½ hour is required. Their resistance to dry heat is so great that a temperature of 150°C. for several hours is necessary to destroy them. Destruction by chemical germicides is difficult, thorough exposure to strong solutions is necessary.

The tubercle bacillus resists drying and retains its virulence in the dried state for months, even when exposed to sunlight. In water it survives longer than in dust. Prolonged cultivation at temperatures above the optimum is said to lessen its virulence in time.

Toxin.—The tubercle bacillus produces a powerful toxin. There is probably both extra- and intracellular toxin formation. The more virulent the bacillus, the more potent the toxin liberated. The intracellular toxin of the tubercle bacillus known as tuberculin is resistant to heat, a temperature of 150°C. being required to destroy it.

Dead tubercle bacilli injected into an animal produce symptoms and lesions indistinguishable from those produced by living bacilli; tuberculin, injected in sufficient amounts, has the same effect.

Tuberculous subjects are very much more sensitive to tuberculin than non-tuberculous subjects, hence the careful administration of tuberculin serves as a diagnostic aid.

The employment of tuberculin in the treatment of tuberculosis is of little, if any, value in the vast majority of cases. Its administration as a therapeutic agent is a highly specialized art and is fraught with danger.

Attempts to immunize healthy animals against tuberculosis by the injection of tuberculin have been unsuccessful.

Sera prepared to combat the disease have failed to do so.

Tuberculins produced by the human tubercle bacillus, the bovine tubercle bacillus and the avian tubercle bacillus are identical in nearly all particulars. A man infected with the human tubercle bacillus is more sensitive to human tuberculin than to bovine tuberculin; a man infected with the bovine bacillus is more sensitive to bovine tuberculin than to human tuberculin.

Agglutinins for the tubercle bacillus may be detected in the blood and serous exudate of some tuberculous animals and not in others; this, in addition to the difficulty of determining reactions, precludes the use of agglutination tests for diagnosis.

Complement fixation tests are not yet applicable to the problems of diagnosis and treatment in this disease. Investigations have shown that complement fixing bodies are present in detectable amount in the blood serum of patients suffering with active tuberculosis and disappear when the disease is arrested, but all efforts up to the present to produce a stable, reliable antigen have been unsuccessful.

Pathogenesis.—Most fatal infections in man are caused by the tubercle bacillus.

Tuberculosis may be a purely localized disease involving only the skin, eye, a bone, an articulation, a kidney or other organ; tuberculosis of the lungs with or without involvement of other parts is by far the most common form of the disease in man. It may be generalized with lesions in many and widely separated parts of the body, with or without bacteria present in the blood.

In man the majority of infections are caused by the human tubercle bacillus, some are caused by the bovine bacillus and perhaps the avian bacillus is the offending organism in rare cases. Ichthic tubercle bacilli probably never infect man.

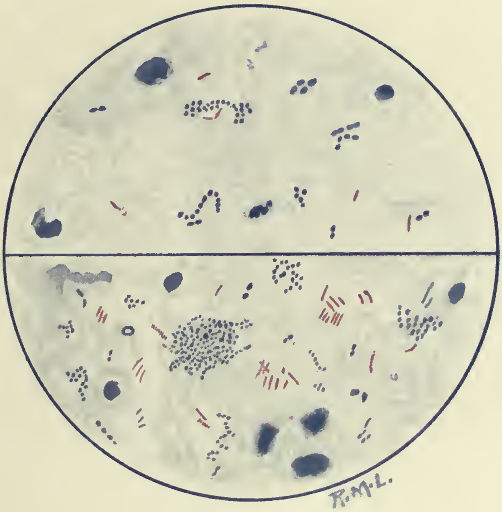

FIG. 19.—TUBERCLE BACILLI IN SPUTUM.

Top half illustrates smear from sputum as expectorated. Bottom half illustrates smear from sputum after centrifugalizing. Stained with carbol-fuchsin and Pappenheim's solution. (4 × eyepiece and 1/12 oil immersion objective.)

Tuberculosis, due to the bovine tubercle bacillus prevails extensively among domestic cattle. Dogs, cats, horses, sheep and goats occasionally are infected.

DIAGNOSIS

Examination of Sputum.—The patient is supplied with a clean, sterile, wide-mouthed container for the collection of the sample. That which is expectorated during the early hours after arising from sleep is most apt to contain tubercle bacilli; hence, the patient is directed to collect it. If expectoration be scant it may be an advantage to collect the output of an entire day.

When received, the sputum is inspected and if pearl-like bodies or particularly dense portions are observed these are selected for examination, as being the most likely portions in which to find bacteria. If the sputum is homogeneous it should be thoroughly shaken before removal of a portion.

The selected particle of sputum is lifted with a sterile platinum loop and placed upon a clean glass slide or cover glass; if fluid it can be readily spread in a thin, even film with the platinum loop; if tenacious or granular an even film is obtained by placing a loopful of sputum on a slide, dropping a second slide on top of it and then drawing the slides apart.

When the sputum has been spread in a thin, even film on the slide or cover glass it is fixed to the slide either by letting it stand in the room until dry, or gently heating over a flame until dry. After fixing by either of these methods it is stained by one of the methods devised to show the acid- and alcohol-fast properties of the tubercle bacillus, previously described.

If examination of slides so prepared fails to disclose the presence of tubercle bacilli it does not necessarily indicate that the sputum is free from them; it merely shows that if present they are scant and not found in all portions of the sputum. Under such circumstances the sputum should be treated as follows:

Add an equal volume of sterile water, shake until homogenized, centrifugalize at high speed for 1/4 hour and make smears of sediment for staining and microscopic examination; or:

Add antiformin to the entire sample of sputum (1 cc. of antiformin to 4 cc. of sputum), mix by shaking and place in incubator for 8 to 24 hours; centrifuge until a precipitate is thrown down, take off the supernatant fluid with a pipette, replace it with sterile water, shake the tubes to thoroughly mix the sediment with the water and spin again until complete precipitation; in this manner wash the precipitate several times; then, smear it on a slide and fix and stain the same as sputum.

Antiformin dissolves practically all the sputum and all bacteria except the tubercle bacillus; with it we can concentrate all the tubercle bacilli that might be present in 50 cc. of sputum into a precipitate of less than 1 cc.

It is to be remembered that a tuberculous person may only occasionally expectorate sputum containing tubercle bacilli. Failure to find tubercle bacilli in sputum from a suspected patient should not be considered significant until samples have been obtained and examined on several consecutive days.

If concentration and repeated microscopic examination of sputum fail to reveal bacilli in a suspected case, a recently obtained sample of sputum should be emulsified with sterile water and injected subcutaneously or into the peritoneal cavity of a guinea-pig. If tuberculosis is caused by such an injection it becomes apparent in 4 to 6 weeks. After such time has elapsed the animal is opened and examined for tubercular lesions.

Examination of Spinal Fluid.—Obtain from 10 to 20 cc. of spinal fluid in a sterile test-tube. If the fluid is clear and has the gross appearance of normal fluid, avoid agitation, place in ice box over night and inspect. The formation of a delicate cobweb-like coagulum suspended in the fluid is indicative of tuberculous meningitis.* This phenomenon occurs only when the fluid is protected from agitation—*i.e.*, transported but a short distance from the patient to an ice box, and that without shaking. The tube must not be disturbed while in the box, and be removed with care.

Spinal fluid may also be examined for tubercle bacilli by centrifugalizing 10 cc. or more of it and making smears of the sediment on slides, staining for tubercle bacilli and examining.

Examination of Urine.—A sterile bottle containing a crystal of thymol is used to collect the urine.

The external genitalia should be washed to remove smega and other bacilli before obtaining the urine, whether it is obtained by catheter or micturition.

Repeated examination may be necessary before tubercle bacilli are discovered.

Morning urine or the output of an entire day may be taken for examination.

It may be centrifugalized in sterile tubes and the entire sediment collected and washed with sterile water once or twice and then spread on slides, dried, stained and examined, or the sample may be treated with antiformin as follows:

Place the urine in a conical glass and add antiformin, a few drops at a time until a light precipitate begins to fall. Cover the vessel and put in a warm place or leave at room temperature over night. Syphon off the supernatant fluid and collect the sediment in sterile centrifuge tubes, wash two or three times with sterile water, spread on slides, dry, stain and examine.

If a sample of 6 ounces or less of urine is to be examined it is best to sediment and wash with water and not use antiformin. Antiformin is of most value when the sediment from a large quantity is to be examined.

When microscopic examinations fail to disclose tubercle bacilli and guinea-pig inoculations are desirable, the sediment from 10 to 30 cc. of urine should be mixed with water and several cubic centimeters injected into the peritoneal cavity.

Examination of Blood.—Under strict aseptic precautions obtain from 10 to 50 cc. of blood from a vein with syringe and needle, add a 50 per cent. antiformin solution, a little at a time and keep in incubator until the blood is entirely destroyed, centrifugalize and wash the sediment with sterile water, spread on slides, stain and mount.

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* Very rarely diseases of the spinal cord or meninges, other than to tuberculosis show this coagulum.

Examination of pleural and other effusions and pus: centrifugalize and make spreads from sediment, dry, stain and examine or inject the entire sediment into the peritoneal cavity of a guinea-pig.

Microscopic examination of these fluids seldom reveals tubercle bacilli even when the tubercle bacillus is the cause of their formation. When injected into guinea-pigs 50 per cent. or more fail to produce tuberculosis.

Examination of Milk.—Centrifugalize 1 pint until cream separates. Remove the cream, dissolve it in ether, centrifugalize the ether, wash the sediment in sterile water and inject it into a guinea-pig, intraperitoneally.

Centrifugalize the skimmed milk until sedimentation is complete and inject the sediment into a second guinea-pig.

Examination of Feces.—Give the patient a dose of salts the night before sample is collected. Add antiformin to the stool and allow it to digest for 12 hours, collect and wash the sediment, inject a couple of cubic centimeters subcutaneously into a guinea-pig and make smears from the sediment for microscopic examination.

DIFFERENTIATION OF

Human, Bovine and Avian types of tubercle bacilli. Recent disclosures suggest that when microscopic examinations of sputum, urine, feces, milk, etc., fail to show tubercle bacilli in suspected cases, cultural tests may do so and it now seems advisable to make such tests. The author has had most promising results with the methods recommended by Petroff, with both Petroff's medium and (William's and Burdick's) modification. They all show practically the same staining characteristics.

Petroff's Method.—Mix equal parts of sputum and 3 per cent. sodium hydroxide solution and incubate at 37°C. for ½ hour. Add normal HCl until neutral to litmus paper (litmus paper used should be sterile). Centrifugalize at high speed for 15 minutes. Pipette off supernatant fluid and plant sediment on Petroff's medium or (William's and Burdick's) modification. Incubate tubes at 37°C. If growth does not appear in 2 weeks examine microscopically as it is frequently present but invisible from the fifth to the fifteenth day.

Observed in tissue the bovine bacillus is shorter and thicker and more uniform in size and staining than the human; the avian longer and relatively more slender, but these differences in size and shape are usually so modified by cultivation on artificial media as to make microscopic differentiation frequently uncertain or impossible.

The human, type grows more rapidly and luxuriantly on culture media than the bovine. In acid glycerin bouillon the human type causes slight if any reduction of the acidity during the early weeks of cultivation, later it makes the medium more acid; the bovine type reduces the acid reaction to or near the neutral point and does not increase acidity later.

The avian type develops best at temperatures too high for the cultivation of the other types.

The bovine type is more virulent for guinea-pigs and rabbits than the human. It kills rabbits in 4 to 6 weeks, while the human type requires several months or more to kill rabbits and frequently fails, too. The avian type is only slightly pathogenic for guinea-pigs, and is virulent for rabbits. The ichthic grows best at room temperature and is not pathogenic for Laboratory animals.

CHAPTER XXI

THE BACILLUS OF LEPROSY, THE SMEGMA AND OTHER ACID-FAST BACTERIA

The bacillus of leprosy, so far as known, infects man only. This is purely a parasitic organism that has not been successfully cultivated outside the human body. It is probable that the leprosy bacillus, deposited in the sputum, nasal secretions, feces and discharges from lesions of the disease, on clothing, utensils, etc., may survive and retain its infectiousness for some time.

The leprosy bacillus is usually present upon the mucosa of the nares from the early stages and throughout the disease. It is present in the enlarged glands, some of the nodules and in the ulcerative lesions of the disease.

When the lungs are involved, sputum may contain them, and when the intestines are affected leprosy bacilli may be found in the feces.

Diagnosis.—Bacteriological diagnosis is based upon microscopic examinations. In every case a sterile cotton swab should be rubbed over the nasal mucous membrane and then rubbed on a clean slide. If ulcers exist smears are made from the necrotic surface and small pieces of the base of the ulcer removed for microscopic examination. If ulceration has not occurred, nodules and enlarged glands are sought for, massaged, and fluid extracted from them and spread on slides.

After drying, the slides are stained exactly as for tubercle bacilli, the leprosy bacillus having the same morphology and staining characteristics as the tubercle bacillus. The lesions of leprosy contain very many leprosy bacilli, some of the tissue cells containing large numbers of them; this is a differential point between leprosy and tuberculosis.

When clinical signs and bacteriological findings furnish inadequate evidence, X-ray examinations of the bones of the feet and hands frequently show characteristic changes.

THE SMEGMA BACILLUS

The smegma bacillus is frequently present upon the external genitalia of both men and women, occasionally on the adjacent cutaneous surfaces and rarely in the mouth or on the tonsils. It is a non-pathogenic saprophyte similar to the tubercle bacillus in many respects and differentiated from it by the following: (1) The smegma bacillus is acid-fast, but not alcohol-fast; Pappenheim's solution, after carbol fuchsin, decolorizes it in less than 20 minutes. (2) The smegma bacillus does not produce tuberculosis when inoculated into guinea-pigs. (3) The smegma bacillus does not produce tuberculin.

There are numerous acid-fast bacteria, including Rabinowitsch's butter bacillus and Moëller's grass bacilli, morphologically indistinguishable from the tubercle bacillus, which occur in air, soil, water, milk, butter and cheese. None of these saprophytes are pathogenic to man. Some are pathogenic to laboratory animals. They are differentiated from the tubercle bacillus as follows:

1. When stained with carbol fuchsin and treated with Pappenheim's solution for 20 minutes they appear blue.
2. They do not produce tuberculin.
3. Many of them grow on plain bouillon, agar, and gelatin and at room temperature. (Their growth, in many respects, resembles that of the tubercle bacillus.)

There has been considerable discussion and disagreement as to the possibility of differentiating the tubercle from non-pathogenic acid-fast bacilli by methods of staining, which should stimulate consideration of the following facts:

First, the vast majority of tubercle bacilli, found in tissue, tuberculous exudates and pus, or directly cultured from them, when stained with carbol fuchsin cannot be decolorized with acids and alcohol within an hour.

Second, occasionally, tubercle bacilli found in tissue, tuberculous exudates pus, or in cultures, are decolorized in a few seconds or minutes with acid and alcohol or with acid alone, after staining with carbol fuchsin.

Third, a very large majority of the non-pathogenic acid-fast bacteria, no matter where found, are decolorized in a few minutes with alcohol, after staining with carbol fuchsin.

Fourth, occasionally non-pathogenic acid-fast bacteria, from various sources, are found which resist decolorization by alcohol as long or longer than the majority of tubercle bacilli—for hours.

CHAPTER XXII

THE COLON BACILLUS

(BACILLUS COLI).

The colon bacillus is a normal inhabitant of the intestinal canal of man and many animals, including all the domestic animals. A large portion of the entire bulk of feces is composed of colon bacilli. The colon bacillus has been found in abundance upon plants and in water of regions barren of animal life.

The colon bacillus is commonly present in air, dust and soil and in water polluted with sewage. From these sources contamination of milk and foodstuffs often occurs.

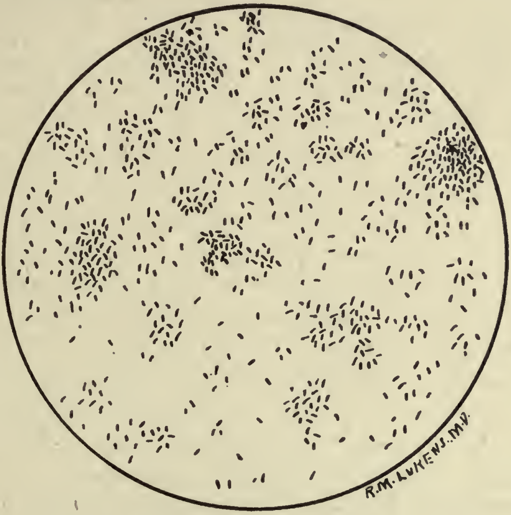

FIG. 20.—BACILLUS COLI. STAINED WITH METHYLENE BLUE.
(4 × eyepiece and 1/12 oil immersion objective.)

Morphology.—The colon bacillus is a straight, round-end bacillus, 2 to 3 μ long and 0.5 to 0.8 μ wide. It has from four to eight lateral flagella, which are about 4 μ long, and is actively motile. Colon bacilli are arranged irregularly, in groups, singly, sometimes in pairs, end to end, and in long filaments.

Staining.—The colon bacillus stains readily with all the usual anilin dyes and is Gram negative.

Growth.—The colon bacillus is aerobic and to a degree anaerobic. It grows at temperatures between 4°C. and 46°C., best at 37°C.

Plain Bouillon incubated at 37°C. begins to show cloudiness in about 12 hours. A light, whitish sediment forms in 24 to 48 hours, which increases in amount for several days. Eventually, if undisturbed, the entire growth precipitates, leaving the fluid clear.

Plain Agar.—In 18 to 24 hours, small, round, moist, whitish-gray colonies appear; these coalesce forming a thin grayish opaque film.

Glycerin Agar.—On glycerin agar the growth has the same appearance, but is slightly more abundant.

Loeffler's Blood Serum.—On Loeffler's blood serum growth is the same as on agar.

Gelatin.—Surface cultures are the same as on agar. Gelatin stab cultures show a continuous yellowish-white growth along the entire length of the stab and on the surface.

Gelatin is not liquefied.

Potato.—On the surface of potato slants a moist, yellowish film appears in 24 to 48 hours; as it ages it becomes thicker and darker, eventually brown.

Milk is acidulated and coagulated in from 12 to 48 hours.

The colon bacillus produces indol. It does not form spores.

The colon bacillus ferments glucose, lactose, saccharose, maltose and levulose, with both gas and acid production.

Resistance.—The colon bacillus is not destroyed by freezing, boiling kills it instantly, moist heat at 60°C. kills it in half an hour. It is destroyed in the hot-air sterilizer in less than an hour at 100°C. Dry, it survives for half a year or more. Exposure to direct sun rays kills the colon bacillus in from 4 to 30 hours. In feces, soil and water, under favorable conditions, colon bacilli remain alive for many months. Resistance to chemical germicides is not great; a 2 per cent. phenol solution will kill the colon bacillus in several minutes.

Toxin.—The colon bacillus produces an intracellular toxin. Filtrates from sterilized heated cultures, injected into animals give rise to the same toxic symptoms and signs as infection with living bacilli.

Agglutinins occur in the blood of infected persons and in the blood of animals immunized against the colon bacillus. These agglutinins are not only specific for the colon bacillus, but show an especially strong action on the particular strain of the organism responsible for their production.

Lysins and Precipitins also occur in the blood of animals immunized against the colon bacillus.

Pathogenesis.—The fact that colon bacilli in enormous numbers are constant inhabitants of the intestinal canal would seem to indicate a lack of pathogenic power of the bacillus or a gradual acquirement of immunity on the part of the host. There are marked variations in virulence, some strains of colon bacilli being sufficiently virulent to cause septicemia and death. As a rule, they are but slightly pathogenic.

In health these organisms are confined to the intestinal canal, but when disease or injury reduces the vitality of the intestinal wall, peritoneum, or any organ adjacent to the intestines, colon bacilli are prone to migrate and attack the impaired organ; thus, they are often found infecting an appendix, gall-bladder, peritoneum, fallopian tube or urinary bladder.

Through causes, and by no means not well understood, colon bacilli migrate to and are often the cause of renal infections, acute, chronic or suppurative.

Rarely they enter the blood-stream and produce a septicemic condition similar to typhoid fever and with similar sequelæ. Colon bacillus infections may not all come from within. This organism can, to a certain degree, produce pus.

Foodstuffs contaminated with colon bacilli are sometimes fermented and split into simpler compounds, some of which are highly poisonous.

When injected into the peritoneal cavity of a guinea-pig or rabbit, virulent colon bacilli cause peritonitis, septicemia and death.

Diagnosis.—Isolation and identification of the colon bacillus, and differentiation from the typhoid and paratyphoid bacilli will be described in a chapter devoted entirely to such procedures.

CHAPTER XXIII

THE TYPHOID BACILLUS

(THE EBERTH GAFFKY BACILLUS)

The typhoid bacillus is present in the blood, urine and feces, sometimes in all three, of typhoid fever patients. It is occasionally present in the urine or feces for weeks, months or years after recovery from typhoid fever. It is variously stated by different observers that from 1 in 500 to 1 in 5000 of the inhabitants of a community are typhoid carriers and that these are responsible for from 20 to 50 per cent. of all typhoid infections. The typhoid bacillus may be found in ulcers, abscesses and other complications or sequelæ of typhoid fever.

From such sources, especially feces, the organism may be transmitted to soil, water, milk, cooking and serving utensils, and various articles of food.

Morphology.—The typhoid bacillus is 2 to 3 μ by 0.5 to 0.8 μ, in size is straight, with rounded ends, and has from 12 to 24 lateral flagella which are about 8 μ long. It is actively motile. The typhoid bacillus has been described as differing from the colon bacillus in the number of its flagella and in degree of motility. These differences are so variable and slight that differentiation cannot be based on them.

Staining.—The typhoid bacillus stains with all the common stains and is Gram negative.

Growth.—Culture of the typhoid bacillus is subject to the same conditions as the colon bacillus. Growth of the typhoid bacillus on plain bouillon, agar and gelatin is indistinguishable from that of the colon bacillus.

Milk is not coagulated and is not acidulated.

Potato.—A typical growth of the typhoid bacillus on potato occurs in 24 to 28 hours. It is very scant, colorless and moist, almost or quite invisible. This typical development does not occur always; there may be no growth or the culture may have the same appearance as a colon culture.

Indol is not produced. There is no spore formation. On media containing any of the sugars the typhoid bacillus does not form gas. Acidulation occurs with glucose but not with lactose nor saccharose.

Resistance.—The typhoid bacillus has the same power of resistance as the colon bacillus to heat, cold, drying, exposure to sunlight and chemical germicides. In water saprophytic bacteria tend to destroy typhoid bacilli so that they usually survive but a few days. Cases have been reported in which typhoid bacilli were found alive and virulent in water after 30 days. In ice they remain alive and virulent for months; also in soil under certain conditions.

Toxin.—The typhoid bacillus produces an intracellular toxin.

Agglutinins occur in the blood of nearly all typhoid patients after the first week of the disease and may be found in the blood of some people weeks, months or years after recovery from typhoid fever. These agglutinins are specific for the typhoid bacillus and their detection forms an important diagnostic test.

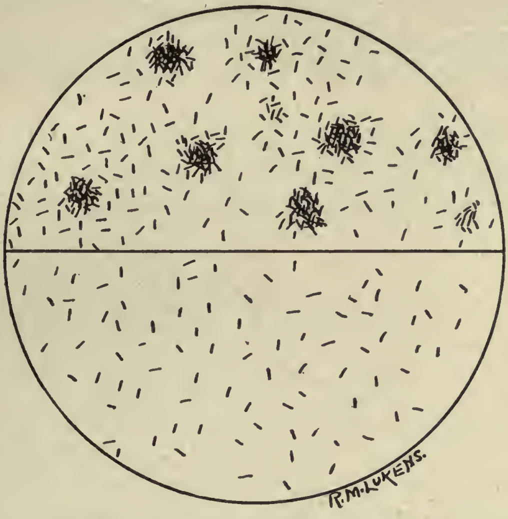

FIG. 21.—TYPHOID BACILLUS.
Upper half of field showing clumps. Lower half of field showing bacilli free from clumps after filtered through paper.

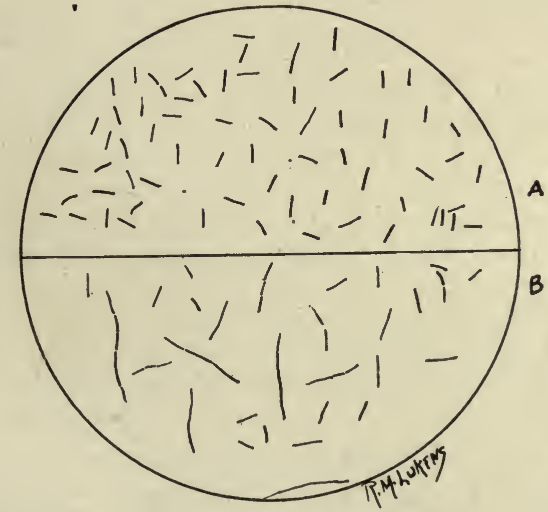

FIG. 22.—TYPHOID BACILLUS.
A. Agar culture, showing a few short filaments. B. Broth culture. Filaments much longer and more numerous. (12 × eyepiece and 1/12 oil immersion objective.)

Specific precipitins, agglutinins and lysins occur in the serum of animals immunized against the typhoid bacillus by inoculation with sterilized bacilli.

Pathogenesis.—The typhoid bacillus is the specific cause of typhoid fever, a septicemia with localized lesions in the small intestine.

During the course of typhoid fever bacilli lodge in the spleen, bone-marrow, gall-bladder and occasionally in other parts; some may survive after subsidence of the fever and remain dormant for months or years and then cause an acute, localized, inflammatory or suppurative condition.

Occasionally the typhoid bacillus has been found the exciting cause of cholecystitis, meningitis and pneumonia without the previous occurrence of typhoid fever.

Apparently, typhoid fever and typhoid infections occur only in man. When virulent typhoid bacilli are injected into lower animals, septicemia and death follow without the manifestations of typhoid fever observed in man.

CHAPTER XXIV

THE PARATYPHOID AND SIMILAR BACILLI

There are a number of organisms that produce manifestations of disease similar to typhoid fever. Some of them closely resemble the typhoid bacillus in morphology, staining and cultural characteristics. Of these the most important are as follows:

PARATYPHOID A BACILLUS.
PARATYPHOID B BACILLUS.
BACILLUS ENTERITIDIS OF GAERTNER.

PARATYPHOID A BACILLUS

The paratyphoid A bacillus produces a clinical picture indistinguishable from a mild typhoid infection or typhoid fever.

The distribution of the paratyphoid A bacillus in the human body is similar to that of the typhoid bacillus. The resistance of these two organisms is practically identical. Pollution of soil, water, etc., and the mode of infection very probably is the same in paratyphoid fever as in typhoid fever.

The morphology, staining, and motility of the paratyphoid A bacillus is indistinguishable from that of the typhoid bacillus. The growth of these two organisms is the same on most culture media.

Milk is not coagulated but is acidulated in from 24 to 48 hours; the reaction remaining constantly acid thereafter. In media containing glucose acid and gas is formed. There is no gas nor acid production with lactose.

Toxin.—The paratyphoid A bacillus forms an intracellular toxin.

Agglutinins are found in the serum of patients and inoculated animals under the same conditions as in typhoid infections. Just as typhoid agglutinins are specific for the typhoid bacillus and do not agglutinate paratyphoid bacilli, so, too, paratyphoid A serum is specific for the paratyphoid A bacillus and does not agglutinate the typhoid bacillus. The specificity of, these agglutinations is limited by the occurrence of group-agglutination reactions as will be explained in the chapter on diagnosis.

PARATYPHOID B BACILLUS

The paratyphoid B bacillus has the same morphology, staining and resistance and, in many respects, the same cultural characteristics as the typhoid and paratyphoid A bacilli.

Unlike them, it infects some of the lower animals, mice, swine and cattle, as well as man. It would seem that infections with paratyphoid B bacillus more often result from the consumption of diseased and contaminated foodstuffs, especially meats, than is the case in typhoid and paratyphoid A infections.

The clinical manifestations of paratyphoid B infections vary. In half or more of the cases the disease runs a course similar to typhoid fever. Frequently the picture is that of a gastro-enteritis of sudden onset, sometimes indistinguishable from true cholera. Rarely the disease has occurred as an acute pneumonia.

The organism may be found in the blood, urine or feces of infected persons and the organism differentiated from other members of the typhoid colon group by cultural, agglutination and complement fixation tests.

Milk is not coagulated by the paratyphoid B bacillus; it is acidulated during the first 24 to 48 hours; in about 10 days the reaction becomes alkaline and remains so thereafter.

Glucose Media show acid and gas production; there is no acid production on lactose or saccharose.

BACILLUS ENTERITIDIS OF GAERTNER

The bacillus enteritidis of Gaertner has been found in diseased mice, guinea-pigs, rabbits and cattle. Infections in man have occurred as a result of eating the meat of diseased cattle. This organism so closely resembles the paratyphoid B bacillus that careful agglutination tests are required to differentiate them.

BACILLUS ENTERITIDIS AERTRYCKE

Bacillus enteritidis aertrycke is closely related to, if not identically the same, as paratyphoid B bacillus.

OTHER MEMBERS OF THE TYPHOID-COLON GROUP

From time to time organisms are found which have the same distribution and pathogenic properties as the colon bacillus; organisms that have also the same morphology, motility and staining characteristics as the colon bacillus, and apparently the same growth on plain agar, gelatin and bouillon, but differ from the colon, the typhoid and other bacilli in their action on carbohydrates and their behavior when brought into contact with sera containing specific precipitins, agglutinins and lysins.

Some of these very probably are distinct species and others, undoubtedly, are atypical strains of colon or similar bacilli which have temporarily lost some of their faculties. So many such organisms have been isolated and named it would be almost impossible to enumerate, much less describe, them and commonly they are referred to as a whole as paracolon bacilli.

In addition to paratyphoid A, B, enteritidis of Gaertner, and enteritidis Aertrycke, each of which is a distinct species, there are a large number of organisms indistinguishable from them and the typhoid bacillus in most properties but failing to conform in some important particular, the exact status of these is doubtful and as a whole they are referred to as paratyphoid bacilli.

For a more elaborate description of the paratyphoid bacilli see Archives of Internal Medicine, vol. v, No. 3, Mar. 15, 1910.

CHAPTER XXV

DIAGNOSIS OF TYPHOID FEVER AND FOOD-POISONING INFECTIONS

The Widal Test.—To carry out this test one must have a 24-hour-old culture of the typhoid bacillus free from clumps. To determine the presence or absence of clumps a loopful of the culture is placed on a cover glass, the cover inverted and placed on a hanging-drop slide so that the fluid is suspended in the well. The preparation is examined under the microscope and if numerous, isolated, actively motile bacilli are observed the culture is fit for use; if bacilli are few or not motile another culture must be procured, and if clumps of bacteria are observed they must be removed before it is fit for agglutination tests. Clumps are removed by filtration through sterile filter-paper.

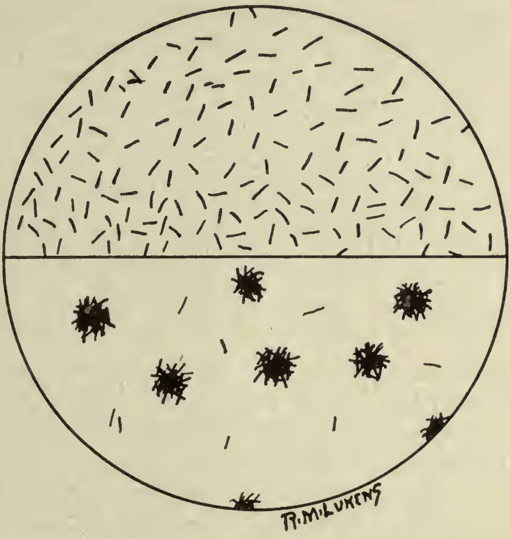

FIG. 23.—WIDAL REACTION.

Upper half of field shows a negative reaction, no agglutination. Lower half of field shows a positive reaction, agglutination of bacilli. (12 × eyepiece and 1/12 oil immersion objective.)

The ear or finger of the patient is cleaned and pricked with a needle or lancet. Several drops to 1 cc. of blood are collected in a sterile capillary tube, a Wright's capsule or upon a clean glass slide. If collected in a tube or capsule the ends are plugged with cotton or otherwise sealed. The blood coagulates in the tube and clear serum separates. When the test is performed the serum alone is used.

If collected on a slide the blood is allowed to dry and when the test is performed several drops of sterile water are mixed with the dried blood and the liquefied whole blood is used.

Having obtained the blood or blood serum and the culture, a hanging-drop slide and several cover glasses are cleaned.

With a sterile capillary or graduated pipette make a 1:20 dilution of the patient's blood or serum, using sterile water. Mix a drop of the diluted serum with an equal quantity of the typhoid culture and place on the cover glass, invert it and place on the hanging-drop slide. Examine under the microscope immediately and again at intervals for ½ hour.

If the bacteria are motile and not clumped when first observed and later form clumps agglutination has occurred and the reaction is positive. If clumping does not occur within ½ hour the reaction is negative.

This test can be made, using dead bacilli in place of the living organisms. As it is more convenient for general practitioners, a preparation giving satisfactory results, known as "Ficker's Typhus Diagnosticum," has been placed on the market.

QUANTITATIVE AGGLUTINATION TESTS

Under strict aseptic precautions 2 cc. to 5 cc. of blood is withdrawn from a vein, allowed to coagulate and the clear serum obtained.

A dozen perfectly clean, sterile, 5 cc. capacity test-tubes are placed in a rack and 1 cc. of sterile salt solution is put into each tube. One-tenth cc. of patient's serum is placed in the first tube and mixed with salt solution; then, 1 cc. of the contents of this tube is transferred to tube No. 2, mixed with the salt solution and 1 cc. is removed from this tube, and mixed with the salt solution in No. 3, and so on until the last tube has been reached. Then, each tube, except the first tube, contains 1 cc. of diluted serum, the dilutions from tube No. 2 to tube No. 12 being as follows: 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120, 1:10,240, 1:20,480, 1:40,960.

One cc. of a 24-hour-old bouillon culture of the typhoid bacillus is added to each tube.

The tubes are incubated at 37°C. for 3 hours, and then examined for agglutination. In this manner the titre limit of the serum, or the highest dilution in which it will agglutinate, is determined.

After incubation, tubes in which agglutination has not occurred have the same appearance as before incubation—the fluid is uniformly cloudy, there is no precipitate. Tubes in which agglutination has occurred contain clear fluid and show a precipitate.

If the macroscopic appearance is doubtful the contents of the tube is poured into a thin, flat dish and examined with microscope, clumps of bacteria being observed if agglutination has occurred.

AGGLUTINATION TESTS WITH LABORATORY SERA

Guinea-pigs and rabbits may be immunized against the typhoid bacillus by intraperitoneal inoculations with the typhoid bacillus given as follows:

First.—Injection ½ cc. of 24-hour-old bouillon culture, heated to 56°C. for ½ hour.

Second.—Injection 1 cc. of same.

Third.—Injection 2 cc. of same.

The first three injections are given at intervals of 48 hours.

Several days after the third injection 0.1 cc. of a 16-hour-old bouillon culture, unheated, is given and several days later 0.5 cc. of a 16-hour-old bouillon culture, unheated, is given. Ten days later the animals are bled and the blood serum collected.

If the procedure has been successful, it will be discovered by agglutination tests that this specific immune serum in high dilutions (1:20,000 to 1:50,000) will agglutinate typhoid bacilli. It will not agglutinate colon, paratyphiod A., paratyphoid B, enteritidis of Gaertner nor other bacilli at all, or only when much less diluted (1:100 to 1:5000).

By immunizing animals in a like manner with paratyphoid A bacillus a specific agglutinating serum for this organism can be obtained; by immunizing with paratyphoid B bacillus, agglutinins specific for it may be obtained. Likewise specific agglutinins for most of the members of the typhoid-colon group of bacteria may be produced.

When an organism of the typhoid-colon group is isolated from a patient's blood, urine, feces, or is found in water or food its nature may be determined as follows:

Make a 24-hour-old bouillon culture of the organism; make dilutions of a specific typhoid serum from 1:100 to the titre limit of the serum. With the suspected culture and known serum make quantitative agglutination tests. If the organism is the typhoid bacillus it will be agglutinated in dilutions up to the titre limit of the serum. If it is not the typhoid bacillus but some other member of the typhoid-colon group it will not be agglutinated in any dilution or only in dilutions much lower than the dilutions in which the same serum agglutinated the typhoid bacillus.

By repeating the experiment with the same culture and a specific paratyphoid A serum, and if necessary again with paratyphoid B serum, eventually we will find a specific serum which agglutinates the organism in the same high dilution as it agglutinates the organism with which the specific serum was produced and the classification is then established.

VALUE OF AGGLUTINATION TESTS

Agglutination tests made with undiluted serum or diluted less than 1:20 are without significance for two reasons:

1. Under such conditions some sera exert an antiagglutinating effect.

2. The sera of some apparently normal non-immune individuals under such conditions will agglutinate one or more of the members of the typhoid-colon group.

The Widal test, made in dilutions of from 1:20 to 1:100, in suspected typhoid fever cases gives accurate results in more than 75 per cent., some claim in more than 90 per cent. of cases; negative if the disease is not typhoid; positive if the disease is typhoid.

The sources of error, other than bad technique, in the Widal test, are as follows:

During the first weeks of typhoid fever the reaction is often negative; in rare cases the reaction is negative until the second or third week of the disease there are a few reported cases in which the Widal test has been negative throughout the disease; occasionally diseases other than typhoid fever give a positive reaction.

Agglutination tests made with specific sera and suspected organisms, when properly performed, give accurate results in the vast majority of cases.

Specific agglutinating sera often contain group-agglutinins; that is, they will agglutinate one or several members of the typhoid-colon group in addition to the particular organism employed to produce them. In practically all cases the power to agglutinate other organisms is much less than the power of agglutinating the particular bacillus which produced them.

Errors can therefore be avoided by determining the highest dilutions in which a serum exerts a group-agglutinating action and those dilutions in which its action is specific.

BLOOD CULTURES

In typhoid fever, paratyphoid, food poisoning, colon septicemia and infections with other members of the typhoid-colon group of organisms in which the clinical course is that of typhoid fever, the offending organism can be obtained from the patient's blood in all cases during the first week or two of the disease and frequently later. In severe prolonged cases of typhoid fever the organism can usually be obtained from the blood later than the second week of the disease.

TECHNIQUE[1]

The night before operation the patient's arm and forearm should be washed with soap and water, alcohol and 1:1000 bichloride, and covered from 4 inches below to 4 inches above the elbow with a moist bichloride dressing and sterile bandage.

A flask containing at least 200 cc. of plain bouillon having a reaction of +1.0 or +1.5 is taken to the bedside. This culture medium must, of course, be sterile. A sterile 10-cc. glass syringe and needle in a sterile container is also required.

The operator scrubs his hands with soap and water and then with alcohol and dries them on a sterile towel. The dressings are removed from the patient's arm and an area of about an inch in diameter over the most prominent vein is painted with tincture of iodine. A crystal of carbolic acid is touched to the spot through which the needle will be inserted. The needle is firmly fastened to the syringe and thrust into the vein. At least 2 cc., better 5 cc. of blood is drawn into the syringe. The needle is withdrawn from the vein, guarded from contamination or contact with anything, and the contents of the syringe emptied into the culture medium without any previous attempt to remove the needle from the syringe.

When the plug is removed from the flask and until it is replaced, contamination of both the plug and culture medium must be guarded against. As soon as the blood is in the flask and the plug replaced, the flask is shaken for several minutes to thoroughly mix the blood and culture medium and to prevent clotting. During the shaking and transportation care must be observed not to bring the contents of the flask into contact with the cotton plug.

The flask is taken directly from the bedside to the laboratory and placed an incubator at 37°C.

After 12 hours the flask is examined for bacterial growth; if not discovered the flask is replaced in the incubator and again examined every 6 hours until growth is observed, or until 72 hours have passed. When the flask remains sterile after 72 hours it is strong evidence that the infection is not due to any of the typhoid colon group of bacteria. Evidence of other infectious disease, especially tuberculosis, should be sought for.

The method of examining the culture for growth is as follows:

Shake the flask, remove the plug, take out a drop of the fluid with a sterile platinum loop, replace the plug, place the drop of fluid on a clean cover glass and suspend on a hanging-drop slide. Examine the drop under the microscope; if no organisms are observed remove and examine several more drops. If a motile bacillus appears it is probably the typhoid, paratyphoid A, paratyphoid B or colon bacillus. To determine, plant a loopful of the culture in litmus milk, make a stab culture in litmus lactose agar and another in litmus glucose agar. Place these tubes in an incubator at 37°C. and, after 18 hours, inspect for characteristic reactions.

Instead of making stab cultures in litmus lactose agar and litmus glucose agar, fermentation tubes, containing litmus glucose bouillon and litmus lactose bouillon, may be planted.

These three subcultures will suffice, in the majority of cases, to disclose the nature of the offending organism. The reaserch may be carried further by making subcultures in Dunham's solution and testing for indol, planting in litmus bouillon containing saccharose, maltose, mannite, levulose, galactose, dulcite, raffinose, arabinose and inulin. Agglutination tests with specific sera may be made and films stained by Gram's method should be examined.

In summing up, it may be stated that in suspected cases of typhoid fever the laboratory diagnosis should consist of both a Widal test and blood culture, with the expectation that one or the other be positive in typhoid; that a negative Widal, together with the occurrence of a motile, Gram negative organism in the culture from the blood is suggestive of paratyphoid or colon infection, especially if occurring after the first week of the disease; that the true nature of the infection can be determined, in most cases, when a culture is obtained from the blood, by making subcultures in three different media, litmus milk, litmus lactose agar or litmus lactose bouillon and litmus glucose agar or litmus glucose bouillon; that repeated failure to obtain either a positive Widal or blood culture in a suspected case of typhoid fever, indicates infection with an organism not belonging to the typhoid-colon group, usually the tubercle bacillus.

EXAMINATION OF URINE

Typhoid bacilli may occur in the urine at times during typhoid fever, in the period of convalescence and occasionally long after recovery, and in carriers, healthy individuals having no history of typhoid fever. Indications for examination of the urine for typhoid bacilli are the occurrence of nephritis or cystitis during the course of typhoid fever or as a sequelæ of the disease, before the discharge of patients recovering from typhoid fever and those suspected of being carriers.

On account of the liability of contamination with colon bacilli, the external genital organs should be thoroughly cleansed before withdrawing urine for examination. The sample should be obtained with a sterile catheter when the bladder is full, and should pass directly from the catheter into a sterile bottle, and be examined without delay.

Different amounts of urine are planted on the surface of separate plates of Endo's medium—from one loopful to several loopsful, and one loopful of the sediment obtained by centrifugalizing 15 cc. of urine. The plants are made by drawing the loop over the medium in the form of the letter Z.

The plates are incubated at 37°C. for 24 hours and then examined for isolated colonies.

Sterile Endo's medium is colorless or a very faint pink Colonies of typhoid and paratyphoid bacilli do not change the color of the medium and themselves appear colorless or whitish. Colon colonies cause the medium surrounding them to turn red and the colonies may appear colorless or pinkish. If typhoid-like colonies appear on Endo's medium, they are removed with a sterile loop and planted in Dunham's solution, litmus milk, litmus glucose and litmus lactose media. The subcultures are incubated at 37°C. for 24 hours and then examined for characteristic changes.

Colon bacillus infections of the bladder or kidneys are common. In suspected cases the urine is examined by planting on Endo's agar and making subcultures as just described.

EXAMINATION OF FECES

Feces always contain large numbers of colon bacilli, which makes isolation of other organisms difficult.

Typhoid bacilli occur in the feces of typhoid fever patients, some convalescents, and occasionally for months or years after recovery from typhoid fever. Typhoid bacilli also occur in the feces of carriers.

Feces should be examined for typhoid bacilli before the discharge of convalescent typhoid patients and when a person is suspected of being a carrier.

The best time to examine feces for typhoid bacilli is immediately after they are expelled from the rectum; attempts made several hours later are usually futile. On account of the great preponderance of colon bacilli in feces it is necessary to first plant the specimen in a medium that will favor the growth of typhoid and paratyphoid bacilli and retard the growth of the colon bacillus. From this primary culture in enriching medium subcultures are made.

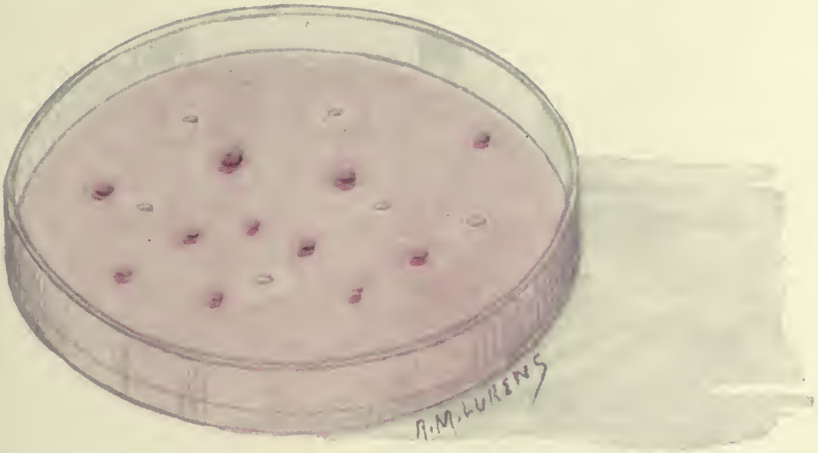

FIG. 24.—PLATE CULTURE, ENDO'S MEDIUM (AGAR) SHOWING TYPHOID AND COLON COLONIES.

The colon colonies appear red surrounded by a red zone in the culture medium. The typhoid are the transparent colonies.

Some observers claim the addition of caffeine (6 cc. of sat. aqueous sol. to 100 cc. of medium) inhibits colon growth without affecting the typhoid.

Crystal violet, Cong. red and brilliant green exert a similar effect.

Teague and Clurman recommend the following enriching medium:

Meat infusion bouillon,	1000 cc.
Gelatin,	100 Gm.
Cong. red (2 per cent. aqueous sol.),	4 cc.
Brilliant green	0.083 per cent.

Tube, 10 cc. to each tube, and sterilize.

Just before inoculation add 1 cc. of the following to each tube:

Bromoform,	20 cc.
Sterile distilled water,	100 cc.

Shake and allow to stand over night. Avoid globules of bromoform on surface and on bottom when removing fluid for addition to media.

Hall in the Berliner klin. Wochenschrift, Dec. 27, 1915, reports excellent results in the isolation of typhoid bacilli from feces by mixing them with benzin or with petroleum-ether and then plating. He states that this will kill all colon bacilli without injuring the typhoid or interfering with their propagation.

TECHNIQUE

The evening before feces are collected for examination the patient is given a dose of salts.

The patient defecates in a clean bed pan or sterile towel.

Liquefy several tubes of Teague and Clurman's medium, add 0.1 cc. of emulsified feces to one tube, shake tube to evenly distribute the feces, transfer 0.1 cc. of the contents of this tube to a second tube, shake second tube to evenly distribute bacteria and transfer 0.1 cc. of its contents to third tube which is also shaken.

Incubate at 37°C. until growth is observed, then at once plate.

Pour Conradi and Drigalski's or Endo's medium into a number of petri dishes and when solidified plant several plates from each tube by removing a loopful of fluid from a tube and drawing it over the surface of one plate after another without recharging the loop. The loop should be drawn over the surface of the agar in the form of the letter Z.

Incubate at 37°C. until growth appears. Examine plates showing discrete colonies and any having the typical appearance of typhoid colonies are removed and planted in plain bouillon; after incubation transplants from the bouillon are made into differentiating media—trypsinized peptone water (for indol), litmus glucose broth, litmus lactose broth and litmus milk.

The method of examining urine and feces for other members of the typhoid colon group is exactly the same as when examining for the typhoid bacillus.

EXAMINATION OF PUS, SPUTUM AND SEROUS EFFUSIONS

When abscess formation, peritonitis, pleurisy, meningitis or pneumonia occurs during the course of typhoid fever or under circumstances which suggest the typhoid bacillus as a probable cause, some of the pus or fluid exudate should be planted on the surface of Endo's agar plates with a platinum loop, incubated, and if typhoid-like colonies appear they are subcultured and examined microscopically.

EXAMINATION OF BILE

A. L. Garbat states that the urine may contain bacilli when the feces and bile do not; that the feces may contain them when the urine and bile do not; that the bile alone may reveal bacteria; that all three—urine, feces and bile—should be examined before typhoid patients are discharged and when an individual is suspected of being a carrier.

He states that the examination of bile for typhoid bacilli is easier than the examination of feces and frequently yields pure cultures of them when examination of feces discloses none. He recommends the following technique:

Have patient swallow an Einhorn duodenal tube when retiring at night; give a liquid breakfast the following morning; 1½ hours after breakfast pour 10 ounces of sterile normal salt solution through tube; ½ hour later withdraw bile from tube with a sterile syringe and plant the contents of syringe in plain broth and on agar; if growth occurs, make subcultures to determine whether typhoid or other bacilli have been obtained.

Bacillus	Size	Motility	Gram's method of staining	Litmus milk
Colon	2 to 3 μ × 5 to 8 μ	Motile	Negative	Acidulated and coagulated
Typhoid	2 to 3 μ × 5 to 8 μ	Motile	Negative	Not acidulated and not coagulated
Paratyphoid A	2 to 3 μ × 5 to 8 μ	Motile	Negative	Acidulated, not coagulated
Paratyphoid B	2 to 3 μ × 5 to 8 μ	Motile	Negative	Acidulated, later turns blue, not coagulated
Gaertner	2 to 3 μ × 5 to 8 μ	Motile	Negative	Acidulated, later turns blue, not coagulated

Bacillus	Glucose	Lactose	Indol	Agglutination
Colon	Acid and gas	Acid and gas	Produces indol	Is not agglutinated with specific typhoid or paratyphoid serum
Typhoid	Acid, no gas	No acid, no gas	No indol	Agglutinated with specific typhoid serum
Paratyphoid A	Acid and gas	No acid, no gas	No indol	Agglutinated with specific paratyphoid A serum
Paratyphoid B	Acid, and gas	No acid, no gas	No indol	Agglutinated with specific paratyphoid B serum
Gaertner	Acid and gas	No acid, no gas	No indol	Agglutinated with specific Gaertner serum

CHAPTER XXVI

DYSENTERY BACILLI

Dysentery is a disease produced by ameba and by a number of closely related bacilli. The bacillary form of the disease occurs sporadically and in epidemics in many parts of the temperate and torrid zones.

The distribution of the various types of dysentery bacilli is not fully known, and it may be the same for each.

Morphology.—The dysentery bacilli have the same size and shape as the typhoid bacillus; most of them are not motile.

In staining, growth in bouillon, agar, gelatin, blood serum and potato they are indistinguishable from the typhoid bacillus; they differ from is and from each other in affect on carbohydrates and agglutination reaction.

HISS AND ZINSSER'S CLASSIFICATION *

Bacillus	Dextrose, levulose, galactose	Mannite	Maltose	Lactose	Saccharose	Dextrin	Litmus milk		
Dysentery (Shiga-Kruse Type)	Acid formed but no gas	No acid, no gas	No acid, no gas	No acid, no gas	No acid, no gas	No acid, no gas	Primary acidity followed by alkalinity	Non-motile	
Dysentery (Hiss-Russel Type)	Acid formed but no gas	Acid, no gas	No acid, no gas	No acid, no gas	No acid, no gas	No acid, no gas	Primary acidity followed by alkalinity	Non-motile	
Dysentery (Flexner Type)	Acid formed but no gas	Acid, no gas	Acid, no gas	No acid, no gas	Acid, no gas	Acid, no gas	Primary acidity followed by alkalinity	Non-motile	
Dysentery ("Rosen")	Acid formed but no gas	Acid, no gas	Acid, no gas	Acid, no gas	No acid, no gas	Acid, no gas	Acid	Slightly motile	Maltose fermented after some days

With few exceptions dysentery bacilli do not form indol. They do not form spores.

Resistance.—Outside the human body the resistance of the dysentery bacilli to germicidal agents is much less than that of the typhoid bacillus.

* "A Text Book of Bacteriology" Hiss and Zinsser.

They cannot be found in feces 48 hours after defecation, due to the antagonistic action of the colon bacillus. In water saprophytic bacteria cause them to disappear in a few days. They dry out in cultures after 3 or 4 weeks, unless transplanted. In a moist state 55°C. kills them in less than an hour. In a hot-air sterilizer they are destroyed in less than an hour at 100°C.

Toxin.—Dysentery bacilli form an intracellular toxin.

Agglutinins, precipitins and amboceptors occur in the serum of dysentery patients and immunized animals. The agglutinins are specific; the serum from a patient agglutinates the type of bacillus causing his condition, but none of the other dysentery bacilli. The amboceptors give a group reaction; they will fix complement when any type of dysentery bacillus is used as antigen.

Pathogenesis—Bacillary dysentery results from ingestion of polluted water or food. The bacteria are usually confined in the primary seat of infection—the intestines—it is possible that in some cases they may migrate to the gall-bladder, but there is not a bacteremia as in typhoid fever. They escape in the feces and bloody mucous flux of the disease. Bacteria are most readily found in the stools during the first days of the disease and disappear after several weeks.

Different types of dysentery bacilli show different degrees of virulence, and it is said that the Shiga type is the most virulent.

Relatively large amounts of living bouillon cultures introduced through the esophagus produce signs and lesions of the disease in guinea-pigs and rabbits similar to the manifestations observed in man.

Diagnosis.—The bacteriological diagnosis of dysentery consists in the examination of feces microscopically and by culture and examination of blood serum for agglutinins.

Feces.—Have the patient defecate into a sterile bed pan, immediately pick out a mucous shred and place it on a slide and let a cover glass fall upon it; then, examine under the microscope for ameba. When ameba are present they may not be found until a number of slides have been examined. If several slides made from mucus fail to show them, slides should be made from the fluid portion of the feces and from solid particles by dipping a camel's-hair brush into it and gently smearing the slide.

After making a microscopic examination for ameba, or before doing so, a shred of the gelatinous-like substance or blood matter found in such stools is sought for, removed with a platinum loop, washed with sterile water and then drawn across the surface of a number of plates of Endo's agar. The plates are incubated at 37°C. for 18 to 24 hours. Red colonies are passed by; any colorless colonies that appear are removed and transplanted into litmus milk, Dunham's solution, and litmus bouillon, containing various carbohydrates, used to differentiate dysentery bacilli. The subcultures are incubated at 37°C. and inspected each day for several days. If a dysentery bacillus is found, a 24-hour-old bouillon culture is used to make agglutination tests with the patient's serum.

Agglutination Tests.—The patient's finger is asepticized, pricked with a sterile needle and several drops or more of blood obtained in a sterile capillary tube. A 1:10 dilution of the serum is made with normal salt solution.

One drop of this 1:10 dilution of serum is mixed with 1 drop of a 24-hour-old bouillon culture of a known dysentery bacillus and placed on a hanging-drop slide and examined for agglutination, as in making a Widal test.

If a negative result is obtained the test should be repeated with as many different types of dysentery bacilli as one may have at hand.

A positive reaction establishes the diagnosis. Negative reactions do not indicate the absence of bacillary dysentery unless made with all the different types of dysentery bacilli. It is to be remembered that agglutinins are not present in the blood during the first few days of the disease and that bacteria cannot be isolated from the stools after the third week of the disease.

CHAPTER XXVII

SPIRILLUM CHOLERÆ ASIATICÆ, VIBRIO CHOLERÆ ASIATICÆ (COMMA BACILLUS)

The cholera spirillum occurs in water polluted with dejecta from cholera patients and from this source probably gets to some articles of food. Epidemics of cholera occur from time to time in all densely populated regions where pollution of drinking water occurs, especially in the temperate zones.

Morphology.—The cholera spirillum usually appears as a curved rod, from 1.5 to 3 μ long, 0.5 μ wide; it is to this common appearance that the descriptive name comma bacillus is due. Long spirals are also observed, having two, three or more curls like a corkscrew. The cholera spirillum has a single terminal flagellum and is actively motile.

Staining.—The cholera spirillum stains with all the usual stains and is Gram negative.

Growth.—The cholera spirillum is aerobic and to a slight degree facultative anaerobic. It grows at temperatures between 15°C. and 40°C., best at 37°C.

Bouillon incubated at 37°C. shows cloudiness in 6 to 8 hours; later, a delicate whitish pellicle forms. In 48 to 72 hours a flocculent precipitate occurs.

Agar.—Round, grayish colonies, with granular centers and smooth edges, appear in 24 hours. Growth on agar is luxuriant and may cover the entire surface with a whitish film in 18 to 36 hours.

Gelatin.–In gelatin stabs growth first appears at the surface of the stab, causing a zone of liquefaction; growth progresses along the stab, more slowly the further from the surface, causing a funnel-shaped tract of liquefaction. Eventually all the gelatin is liquefied.

On gelatin plates yellowish, granular, irregular colonies, surrounded with a more highly refractile, whitish zone appear in 24 hours.

The medium is liquefied.

Potato shows an abundant, clear, brownish growth if the medium is alkaline. On neutral or acid potato, growth is scant or absent.

Milk is coagulated by some and not coagulated by other strains of the cholera spirillum.

Indol is nearly always produced.

There is no spore formation.

Fermentation of all the sugars in common use occurs.

Dunham's solution is especially favorable for the cultivation and isolation of the cholera spirillum. Incubated at 37°C., growth accumulates at the surface in 6 to 12 hours.

Resistance.—The cholera spirillum is less resistant to germicidal agents than the typhoid bacillus. Freezing does not injure it. Drying rapidly destroys it. In a moist state exposure to 55°C. kills in ½ hour. In feces and in water containing saprophytic bacteria they are destroyed in a few days.

Cholera vibrio survives in bay water 7 to 49 days but will not multiply in it.

Toxin.—The cholera bacillus produces an intracellular toxin or toxins.

The injection of living or dead cholera organisms into the subcutaneous tissues, peritoneal cavity or into a vein produces immunity. The serum of immunized animals contains agglutinins and amboceptors for the cholera spirillum.

Agglutinins also occur in the serum of many cholera patients.

Pathogenesis.—Different strains of the cholera spirillum show different degrees of virulence. The cholera spirillum enters the body through the alimentary canal and lodges in the intestine; other tissues are not invaded, but the toxin is absorbed and passes into the general circulation. The infecting organisms are discharged in the feces where they may be found in abundance. The spirillum may also be found in the rectum and feces of carriers.

While cholera is a disease of man exclusively, it can be simulated by artificial means in animals.

Virulent cultures of the cholera spirillum injected into the peritoneal cavity of a guinea-pig usually cause peritonitis, toxemia and death.

Diagnosis.—There are so many vibriones found in water, and occasionally in feces, which are indistinguishable from the cholera spirillum in morphology, staining, growth on culture media and results from animal inoculation tests, that identification of the cholera spirillum necessitates agglutination tests and is often difficult.

Isolation of the cholera spirillum from other organisms, occurring with it in water and feces, is favored by the luxuriant and more rapid growth of the cholera spirillum in certain liquid and solid media, notably Dunham's solution. A valuable review of these selective media may be found in Hygienic Laboratory Bulletin, No. 91, 1913, U. S. Public Health Service.

The stools of cholera patients contain many cholera organisms and should be examined immediately after passage as follows:

Technique.—Take a loopful of the dejecta and place in a tube containing 10 cc. of Dunham's solution. Incubate the tube at 37°C. for 6 hours. Remove a loopful of the culture from the top of the medium and draw the loop across the surface of several plates without recharging it. Plain agar, plain gelatin or Dieudonne's alkaline blood may be used for plating. Incubate agar plates at 37°C. and gelatin plates at 22°C. to 25°C. After 16 hours examine plates and remove any colonies that appear to be cholera spirillum; transplant into Dunham's solution, incubate at 37°C. for 6 or 8 hours, remove a loopful and make a hanging drop preparation. Examine with the microscope and if a motile spirillum is found in pure culture, make transplants into milk on plain agar and into Dunham's solution. Incubate for 24 hours at 37°C. Test the Dunham's solution for indol and the agar growth for Pfeiffer's reaction as follows: Scrape half the 24-hour-old growth off the agar and emulsify in 2 cc. of sterile bouillon, make a hanging-drop and see that the emulsion is free from clumps and that the organisms are motile. Inject the 2 cc. of emulsion into the peritoneal cavity of a guinea-pig that has been immunized against the cholera spirillum. Ten minutes later withdraw several drops of the peritoneal fluid, place on hanging-drop slide and examine with the microscope. If the organism injected into the pig was the cholera spirillum it will have lost its motility and will appear as granular dots. If it was not the cholera spirillum it will appear unaltered, morphology and motility the same as before injection.

The isolation of the cholera spirillum from water will be described in the chapter devoted to the examination of water.

CHAPTER XXVIII

MICROCOCCUS MELITENSIS

Micrococcus melitensis, first found in Malta, has been reported as occurring in many regions adjacent to the Mediterranean, also in China, Central Africa and England, and may have a still wider distribution.

This organism is found in the soil of regions where the disease prevails and in the milk of infected goats and the urine of infected patients.

Morphology.—The micrococcus melitensis is a small, round or oval organism arranged singly, in pairs and short chains, the latter often parallel to each other. According to early observers this organism is a coccus. Recent authorities classify it as a bacillus. It is a non-motile, stains with the usual anilin stain and is Gram negative.

Growth.—Micrococcus Melitensis is an obligate aerobe and grows best at 37°C.

Bouillon.—Incubated at 37°C. shows cloudiness in 3 to 5 days.

Agar.—Small white colonies appear in 3 to 5 days. Growth is most abundant on glycerin agar, even on this medium it is scant.

Gelatin.—No apparent growth occurs on this medium, it is not liquefied.

Potato.—Does not show growth.

Milk is not acidulated and is not coagulated.

Indol is not formed, spores are not formed, sugars are not fermented.

Resistance.—Micrococcus melitensis is somewhat more resistant to the various germicidal agencies than the typhoid bacillus.

Toxin.—Toxin production in culture is slight.

Agglutinins.—Specific agglutinins occur in the blood of immunized animals.

The saliva, blood, and sometimes the urine, of infected persons will agglutinate the micrococcus melitensis in low dilutions after the first week of the disease.

Pathogenesis.—The micrococcus melitensis produces a disease in man known as Malta fever or Mediterranean fever, a condition simulating typhoid fever or malarial fever. The majority of cases result from drinking the milk of goats infected with the micrococcus melitensis; some result from contact with patients, especially when handling urine, as in nursing. It is possible that infection may be acquired from other sources, as dust and soil.

Diagnosis.—Diagnosis is based on the discovery of the organism in the urine and its agglutination with the serum of an immunized animal.

After the first week of the disease the patient's serum may be tested, in dilutions of 1:10, 1:20 and 1:40 for agglutinations.

CHAPTER XXIX

BACILLUS PYOCYANEUS

The bacillus pyocyaneus is primarily a saprophytic organism and occurs in dust, soil and water and substances contaminated by them.

Morphology.—Bacillus pyocyaneus is 1 to 2 μ long and 0.5 μ wide, sometimes as in old broth cultures elongated forms are observed. It is arranged singly and in short filaments and is actively motile.

Staining.—It stains with all the usual anilin stains and is Gram negative; old cultures (involution forms) may show granular or irregular staining.

Growth.—Bacillus pyocyaneus is an aerobic and facultative anaerobic organism growing at temperatures between 18°C. and 43°C., best at 37°C. Cultivated under aerobic conditions, it produces pigment-pyocyanine. This pigment diffuses through the medium containing the bacilli and gives it a greenish or bluish tint. This pigment can be separated into two or three portions as follows:

To a bouillon culture showing color add about one-tenth its volume of chloroform, shake, allow to stand at rest until chloroform precipitates. The chloroform is a deep blue and the upper part of the bouillon shows a fluorescent green.

Bouillon.—Incubated at 37°C. becomes cloudy in 12 hours; then pigment begins to appear, at first at the surface, later throughout the medium. After several days a thick, whitish pellicle forms on the surface; later an abundant precipitate is thrown down and the medium changes from greenish-blue to yellowish-brown.

Agar.—Growth appears in 24 hours and covers the entire surface in several days and the agar attains a greenish color.

Gelatin surface cultures after 48 hours at 20°C. to 25°C., show small, round yellowish granular colonies; later the dense central portion of the colonies is surrounded by a thin, granular filamentous zone. Liquefaction begins around the colonies and rapidly involves the entire medium. The gelatin is tinted like agar.

Gelatin.—Stab cultures show growth along the stab in 48 hours. The gelatin is tinted green and liquefied.

Potato.—A thick brown surface growth develops in 2 or 3 days.

MacConkey's bile-salt medium shows gas formation and a decided greenish tint in 24 to 48 hours.

Bacillus pyocyaneus does not form spores.

Toxin.—Bacillus pyocyaneus forms an intracellular toxin capable of producing effect similar to that caused by the living bacillus.

Agglutinins are present in the blood of immunized animals.

Resistance.—Bacillus pyocyaneus is somewhat more resistant to heat and chemical germicides than the typhoid bacillus. Soil seems to be its normal abode and it survives in water longer than either the typhoid or colon bacillus.

Pathogenesis.—Bacillus pyocyaneus, present in eggs, milk, ice cream and other articles of food, for a long time before consumption, can produce changes which result in acute gastroenteritis or toxemia when such food is eaten.

It may produce gastro-enteritis especially in debilitated children.

Bacillus pyocyaneus is frequently present upon the skin and occasionally in the mouth or nose of healthy people without causing ill effect. Healthy individuals seem to be immune to this organism. But when injury or infection with some other organism reduces the vitality to a marked degree, then bacillus pyocyaneus may enter the devitalized part and further aggravate the condition. Thus, it is most commonly found in chronic suppurative conditions, in advanced phthisis and extensive, grossly infected wounds.

Diagnosis.—Pus containing bacillus pyocyaneus usually has a distinct greenish color which suggests its presence. If the pus is smeared on a slide, fixed by heat and stained by Gram's method and a short, slim, Gram negative bacillus found it is sufficient to indicate the presence of bacillus pyocyaneus.

More exact identification may be obtained by inoculating bouillon, agar or gelatin and observing the pigment formation. It should be remembered in this connection that cultures direct from tissue or pus sometimes do not show pigment production until subcultured once or twice; that pigment appears only under aerobic conditions.

CHAPTER XXX

BACILLUS PROTEUS VULGARIS

Bacillus proteus vulgaris is one of the common organisms of putrefaction. It occurs in air, soil and water, and is present wherever putrefaction takes place. It splits proteids into their simplest components.

In morphology, motility and staining it is indistinguishable from the colon bacillus. It is differentiated from the colon bacillus by the following characteristics:

Optimum growth at 25°C.

Liquefies gelatin and blood serum.

In milk it causes coagulation and then liquefies the clot.

It is agglutinated with the serum of immunized animals.

Toxin.—Bacillus proteus vulgaris produces little, if any, toxin.

Pathogenesis.—This organism is not pathogenic for man, but is of importance on account of the frequency with which it contaminates and putrefies meat and other putrescible foods exposed to air and stored at room temperature. In the process of putrefaction it liberates compounds, some of which are toxic.

CHAPTER XXXI

BACILLUS LACTIS AEROGENES AND BACILLUS BULGARICUS

Bacillus lactis aerogenes is widely distributed in air, water and soil. It is almost constantly present in milk and the intestinal canal of man. This organism is the chief cause of souring of milk. It produces large quantities of lactic acid and can survive in media which it has made strongly acid.

Escherich described this organism as a distinct entity and some still consider it such. At the present time many hold the view that bacillus lactis aerogenes and bacillus Friedländer are two different organisms belonging to a numerous group the members of which are closely related in morphology, growth on culture media, etc.

"No valid distinction can now be drawn between the pneumobacillus and the bacillus described by Escherich as the bacillus lactis aerogenes; the proof of their identity was sketched by Denys and Martin and extended by Grinbert and Legros. These researches were confirmed by Bertarelli; he considered the bacillus lactis aerogenes to be merely a variety of pneumobacillus" (Besson).

BACILLUS BULGARICUS

Bacillus Bulgaricus, originally isolated from and identified as the dominating organism in sour milk, in Bulgaria, is a harmless, non-pathogenic organism said to differ from other saprophytes found in milk in the following particulars:

First, it does not putrefy milk; second, it produces, in milk, a greater amount of lactic acid, in a shorter time, than other bacteria; third, it does not produce changes in milk that injure its food value.

When ingested alive, the bacillus Bulgaricus is supposed to survive a sufficient time to produce enough lactic acid to destroy putrefactive bacteria and thus preclude or arrest deleterious putrefaction in the intestinal canal of man.

Morphology.—Four to 8 μ long, 0.5 to 1.0 μ wide, arranged singly, in pairs, end to end and in filaments; sluggish motility.

Staining.—Stains with the usual anilin dyes. Young cultures are Gram positive; older cultures show both Gram positive and negative organisms and some which show granular staining with methylene blue.

Growth.—Bacillus Bulgaricus is an aerobic and facultative anaerobic organism growing best at 42°C. It grows poorly or not at all in plain bouillon, agar and gelatin.

In milk, whey, whey-bouillon and whey-agar, abundant growth occurs in 24 hours, with an abundant production of lactic acid. In a few days the media become so acid the bacilli are killed by it.

In media favorable for its growth bacillus Bulgaricus will remain alive for more than 9 months if kept at 15°C.

CHAPTER XXXII

BACILLUS BOTULINUS

Bacillus botulinus has been found in various meat products, especially sausage, less frequently in eggs, cheese, butter and beans.

Morphology.—Four to 8 μ long, 0.5 to 1 μ wide, with rounded ends, arranged singly, in groups and short chains. It is said to be motile.

Staining.—Stains with all the usual stains and is Gram positive.

Growth.—Bacillus botulinus is an obligate anaerobic organism growing best at 25°C. to 30°C. It grows best in meat, especially pork.

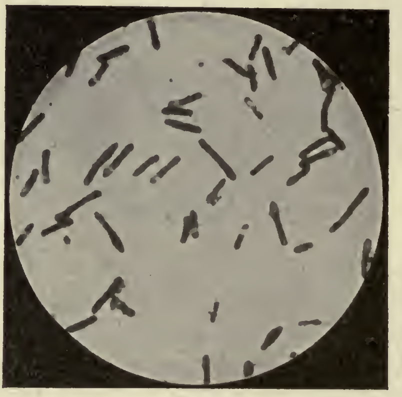

FIG. 25.—BACILLUS BOTULINUS.
Some individuals containing spores. (*After van Ermengen.*)

Media containing glucose show gas formation, gelatin is liquefied, milk is not coagulated.

Bacillus botulinus forms spores which are situated near the extremity of the bacillus and are slightly wider than it, causing a bulge. Spores are killed in a short time at 80°C. in a moist state. Bacillus botulinus cannot survive in brine containing more than 6 per cent. of salt.

Toxin.—Bacillus botulinus produces a powerful extracellular toxin which accumulates in sausages, meat pies, etc., containing the organism. A temperature of 100°C. destroys this toxin.

Pathogenesis.—Bacillus botulinus does not infect man, but when food containing its toxin is eaten the toxin is absorbed and acts as a powerful poison, causing death in 25 per cent. of cases. Following the ingestion of toxin a period of incubation, never less than 6 hours, elapses before the onset of symptoms. Impairment of vision, disturbance of speech, increased saliva-tion, general muscular weakness, and, in severe cases, paralysis are constant signs.

Postmortem degeneration of ganglion cells of the anterior horn and bulbar centers is found.

A period of incubation, lasting about 24 hours, elapses after the toxin is ingested before symptoms develop.

Diagnosis.—Bacteriological diagnosis can only be made by finding the organism or its toxin in food. Cats and dogs fed on meats, etc., containing the toxin, or injected with 3 cc. of a bouillon culture are made ill, present characteristic signs and frequently die.

CHAPTER XXXIII

BACILLUS AEROGENES CAPSULATUS

B. PERFRINGENS, B. EMPHYSEMATIS VAGINÆ, B. PHLEGMONIS EMPHYSEMATOSÆ OR B. WELCHII

Bacillatus aerogenes capsulatus occurs in air, dust, soil and water; it is frequently, if not constantly, an inhabitant of the intestinal canal of man and animals.

Extensive studies would seem to indicate that this organism is frequently present in the gall-bladder, liver and blood of apparently healthy normal dogs and in the liver or blood of some apparently healthy people. It is not uncommon to find the bacillus aerogenes capsulatus in blood cultures, and cultures from diseased joints in cases of rheumatism and arthritis deformans, even when it is demonstrable that bacillus aerogenes capsulatus is not the cause of the arthritis.

This organism is frequently found in water polluted with sewage.

Morphology.—B. aerogenes capsulatus is a large bacillus, 3 to 8 μ long, has ends which are square or slightly rounded, is non-motile and is arranged singly and in chains. Organisms removed from tissue, especially blood, usually show a distinct capsule; in culture the capsule may disappear.

Staining.—It stains with all the common stains and is Gram positive.

Growth.—Bacillus aerogenes capsulatus is an obligate anaerobe and grows best at 37°C.

Bouillon incubated at 37°C. shows marked cloudiness in 12 to 24 hours; later an abundant whitish flocculent sediment forms.

Agar.—Within 18 to 24 hours round, grayish colonies develop. They vary in size from 2 millimeters to 1 centimeter in diameter, are flat and have irregular margins.

Stab cultures in agar show gas formation.

Gelatin.—Growth on this medium is the same as on agar. Gelatine is slowly liquefied in the majority of cases; occasionally liquefaction does not occur.

Milk is rapidly acidulated and coagulated.

Potato.—Slight growth without any characteristic appearance occurs on potato.

Glucose, lactose and saccharose are fermented; gas is produced from roteids.

Bacillus aerogenes capsulatus forms spores.

Toxin.—There is little, if any, toxin production.

Resistance.—Bacillus aerogenes capsulatus is somewhat more resistant to chemical and thermal germicides than the typhoid bacillus. Spores are highly resistant to chemicals and require boiling for 1 hour to kill.

Pathogenesis.—Bacillus aerogenes capsulatus may cause emphysematous gangrene when it gains entrance into body tissue at the site of an injury. It probably is capable of producing gastro-intestinal disturbances. Relatively, the pathogenic power of bacillus aerogenes is slight; several cubic centimeters of a bouillon culture injected into the circulation of a rabbit frequently fails to produce ill effect. Guinea-pigs seem most susceptible, yet some strains are not pathogenic for these animals.

Diagnosis.—The morphology, and character of growth in agar and gelatin stab cultures, is usually sufficient to establish the identity of this organism. When further studies are desirable and when the bacillus is found, associated with other organisms and needs be isolated, a bouillon culture is made. Three to 5 cc. of an 18-hour-old culture is injected, intravenously, into a rabbit. About 15 minutes after inoculation the rabbit is killed with ether or chloroform and placed in an incubator at 37°C. Twelve to 18 hours later the rabbit will appear much distended with gas. Crepitation is felt in the subcutaneous tissue due to accumulation of gas. If the peritoneal cavity is opened by puncture and a flame touched to the escaping gas it will burn with a blue flame. Cultures made from the blood, liver, heart and other organs will yield pure cultures of the bacillus aerogenes capsulatus.

CHAPTER XXXIV

BACILLUS MALIGNI ŒDEMATIS[1]
BACILLUS OF MALIGNANT EDEMA OR VIBRION SEPTIQUE

The bacillus of malignant edema is commonly present in garden soil, barnyard manure and in river silt.

Morphology.—It is a large bacillus, 3 to 10 μ long, 0.5 to 1.0 μ wide, has slightly rounded ends, occurs singly and in chains and under anaerobic conditions is slightly motile.

Staining occurs readily with the usual anilin dyes and it is Gram positive.

Growth.—The bacillus of malignant edema is an obligate anaerobe and grows at temperatures between 15°C. and 41°C., best at 37°C.

Bouillon shows cloudiness in 12 to 18 hours; after 24 to 48 hours a heavy, whitish sediment forms and the bouillon above becomes clear.

Agar.—Colonies develop on the surface in several days; they are small, round, elevated and grayish.

Stab Cultures in Agar show growth in 1 or 2 days. It appears at first as a white line along the stab and then extends laterally, giving the medium a grayish cloudy appearance and forming gas, which splits the agar.

Gelatin.—Growth on gelatin has the same appearance as on agar. The medium is rapidly liquefied.

Blood serum is liquefied.

Milk is slowly coagulated.

Potato shows slight, if any, growth.

The bacillus of malignant edema produces indol and forms spores.

Resistance.—Bacilli are destroyed in a moist state in less than an hour at 70°C. They are more resistant to chemical germicides than the typhoid bacillus. Spores are very resistant to all germicidal agents and require boiling for at least 1 hour to kill them.

Toxin.—The bacillus of malignant edema forms a small amount of a weak intracellular toxin.

Agglutinins.—Specific agglutinins occur in the blood of immunized animals.

Pathogenesis.—This organism rarely infects man. Being an obligate anaerobe infection is most apt to occur in puncture and deep lacerated wounds and has been most frequently observed in severe compound fracture wounds grossly contaminated with dirt.

Most of the domestic and laboratory animals are susceptible to the bacillus of malignant edema.

Localized edema at the point of inoculation, grave septicemia, convulsions and death are observed in all animals subject to infection.

Diagnosis.—The bacillus of malignant edema is easily recognized by injecting a small amount of fluid suspected of containing the organism into a guinea-pig, subcutaneously, noting the effect and making microscopic and cultural examinations of bacteria found in the blood or œdematous area when the pig dies.

CHAPTER XXXV

BACILLUS TETANI

(TACK BACILLUS)

The bacillus tetani is frequently present in dust and soil, especially dust accumulated in old buildings, farms and cattle sheds and in soil enriched with horse and cow manure.

It is almost constantly present in the intestinal contents and feces of sheep—the source of cat-gut used in surgery.

The comparative frequency with which tetanus has followed black powder burns, would seem to indicate some relation between this substance and bacillus tetani.

Morphology.—It is a straight bacillus 3 to 5 μ long, 0.3 to 0.6 μ wide, has rounded ends, is slightly motile and produces spores. While most spore-bearing bacteria carry their spores in or close to the middle, the spores occur in tetanus bacilli at one end. As they are somewhat larger in diameter than the bacillus itself and cause a bulging, a characteristic tack-like appearance is produced. Tetanus bacilli are arranged singly and in irregular clumps of two, three or four organisms.

Staining.—Bacillus tetani stains with all the usual anilin stains and is Gram positive.

Growth.—Bacillus tetani is primarily an obligate anaerobe; under certain conditions it is also facultative aerobic. Growth occurs at temperatures between 15°C. and 44°C., best at 37°C.

Bouillon incubated at 37°C. under anaerobic conditions becomes cloudy in 18 to 36 hours; later a grayish sediment forms and after a week or two the medium becomes clear. An offensive odor, suggestive of burnt horn, is produced.

Agar.—Small, spherical, whitish colonies appear in 36 to 48 hours. They have a dense center surrounded by a thin, irregular zone.

Stab cultures show a cloudy appearance of the medium, which is split by gas formation.

Gelatin.—Surface growth is similar to that observed on agar.

Stab cultures in gelatin, as in agar, at first show fine white lines of growth extending at right angles to the stab; as these increase the medium becomes cloudy. Gelatin is slowly liquefied, and when liquefaction is complete a whitish sediment is deposited and the medium becomes clear.

Blood serum shows nothing characteristic and is not liquefied.

Potato.—The growth of the tetanus bacillus on potato is slight, glistening and colorless.

Milk is not coagulated.

Bacillus tetani produces indol, carbon dioxide and hydrogen sulphide.

Resistance.—Bacillus tetani is slightly more resistant to germicidal agents than the typhoid bacillus, but its spores are very resistant. They survive in 5 per cent. phenol and 1 : 1000 bichloride solutions at 20°C. for hours. Sterilization in a hot-air sterilizer sometimes requires a temperature of 150°C. for several hours. Boiling is said to destroy tetanus spores in 10 minutes, but frequently boiling for an hour or more is necessary. Steam at 15 pounds pressure destroys them in a few minutes.

Direct exposure to sunlight and air rapidly alters tetanus spores and in the course of 10 to 15 days destroys them. Spores upon the surface of the ground where air and light strike them die in one or several weeks. Upon wood, in the dust and crevices of buildings, where they are protected from light and, to some degree from air, they remain viable and virulent for years.

Toxin.—Bacillus tetani produces a powerful extracellular toxin. The symptoms and fatalities of tetanus are caused by the filterable toxin called tetanospasmin. It seems to have an especial affinity for nerve tissues and to travel to the central nervous system along the motor tracts.

"Tetanolysin" is a separate extracellular toxin produced by the tetanus bacillus. It causes hemolysis of the red blood cells of many animals.

By repeated injection of sublethal doses of toxin, animals can be immunized against tetanus and the serum of such animals is highly antitoxic.

Agglutinins are not present in the blood of those infected with bacillus tetani.

Pathogenesis.—Tetanus is a disease having a high mortality. Man and the horse are especially susceptible; guinea-pigs, mice and rats are very susceptible, rabbits less so; dogs possess a considerable degree of immunity.

Absence of oxygen, devitalization of surrounding tissue and presence of other organisms are conditions most favorable to infection, hence the disease is usually seen following puncture wounds, extensive traumatism and deep lacerations grossly soiled with street dust, soil or cinders. Bacillus tetani lodges at the point of entry; it does not enter the blood-stream or lymph-stream and does not pass to other parts of the body. The invading organisms are strictly localized and the extracellular toxin they liberate travels along the course of the motor nerves to the motor centers.

It has been fairly well established that tetanus spores may enter the body and remain dormant at the atrium of infection for weeks, eventually being destroyed or removed from the body through the action of natural protective forces or as the result of injections of tetanus antitoxin—or, after remaining dormant for a number of days or weeks, become active, producing bacilli, toxin and the characteristic phenomena of tetanus. Such activity after quiescence is favored by a local or general weakening of the natural or normal immunizing forces as commonly follows trauma, intercurrent infections and certain chemical intoxications. It has been known for a long time that quinine is an activator of tetanus spores and bacilli; that the administration of small or large doses of quinine favors the development of tetanus.

Diagnosis.—For the bacteriological diagnosis of tetanus, pus or fluid is removed from any wound that may exist, smears are made, fixed, stained and examined microscopically; tetanus bacilli may or may not be observed. Agar and bouillon are inoculated with the pus, two tubes of each; half of these are incubated under aerobic and the others under anaerobic conditions at 37°C. for 24 to 48 hours and then inspected with the naked eye and smears made for microscopic examination.

Such examinations not infrequently fail to disclose tetanus bacilli or spores that are present and, therefore, bacteriological examinations are not depended upon in making a diagnosis of this infection.

CHAPTER XXXVI

BACILLUS ANTHRACIS

Bacillus anthracis occurs in soil and on grasses of pasture lands traversed by infected animals and land polluted by the burial of animals dying with anthrax. It also occurs upon wool and hides of animals coming from districts where the disease prevails.

Morphology.—Bacillus anthracis is a large, straight non-motile rod with square ends, 5 to 10 μ long and 1 to 2 μ wide.

Bacilli observed in tissue are arranged singly and in pairs, end to end, and do not contain spores. Those obtained from cultures are arranged in long filaments and show spore formation. The spores form in the middle of the bacilli.

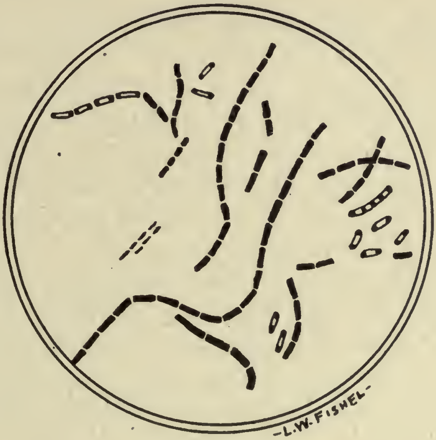

FIG. 26.—BACILLUS ANTHRACIS.
Some showing spores. (4 × eyepiece and 1/12 oil immersion objective.)

Staining.—Bacillus anthracis stains readily with the usual stains and is Gram positive. The spores require special methods to tint them.

Growth.—Bacillus anthracis is an aerobic and facultative anaerobic organism. It grows at temperatures between 15°C. and 45°C., best at 37°C.

Bouillon incubated at 37°C. shows growth in 24 hours, a whitish pellicle forms on the surface and a stringy, whitish sediment collects in the bottom. When at rest the medium remains clear.

Agar.—Round, irregular-edged, flat, opaque, whitish colonies appear in 18 to 36 hours and tend to coalesce, forming a pellicle covering the surface.

Gelatin.—On the surface of gelatin growth appears as on agar.

Gelatin stabs show growth along and radiating from the stab described as "inverted fir-tree growth." Gelatin is slowly liquefied.

Potato.—A dry, whitish pellicle forms on the surface.

Milk is acidulated and coagulated.

Resistance.—Bacillus anthracis is only slightly more resistant to heat and chemical germicides than the typhoid bacillus.

The spores are very resistant to all germicidal agents; exposure to strong chemicals, direct sunlight and drying is withstood for a long time. An exposure of several hours at 150°C. to 200°C. is required to destroy spores in a hot-air sterilizer; boiling for 1 hour sometimes fails to kill them; steam under 15 pounds pressure destroys anthrax spores in a few minutes.

Toxin.—Bacillus anthracis produces no extracellular toxin.

Agglutinins and lysins are not demonstrable in the blood of either infected or immunized animals.

Pathogenesis.—Many domestic animals are very susceptible to anthrax, usually contracting the infection through eating from pastures contaminated with anthrax spores. The feces from such animals contains spores. Minute amounts of bacillus anthracis or spores injected into rabbits or guinea-pigs produce death.

In man infection is almost entirely confined to those engaged in handling hides, wool or cattle.

Wool sorters are usually infected through the respiratory tract and suffer pulmonic involvement, followed, in most cases, by bacteremia and death. Those who handle hides are usually infected through abrasions of the skin, a lesion known as malignant pustule developing at the point of inoculation. The infection may remain localized or the bacteria may enter the blood-stream. The mortality is high—25 per cent. or more.

Infection through the alimentary canal in man is exceedingly rare.

Diagnosis.—When the disease is localized bacteria may be obtained from the pustule and adjacent tissue; if bacteremia occurs organisms may be obtained from any blood-vessels—even capillaries are clogged with them.

Bacillus anthracis is easily identified by its morphology and growth on plain agar, gelatin or bouillon.

CHAPTER XXXVII

BACILLUS SUBTILIS

(HAY BACILLUS)

Bacillus subtilis is present in soil, dust, air and almost constantly upon hay. It is not a pathogenic organism, but may enter chronic, neglected wounds or ulcers and exist there as a saprophyte. Frequently is it found in contaminated milk, blood serum, infusions and laboratory culture media. It is a spore-forming bacillus and these spores are very resistant, withstanding boiling for 5 or 10 minutes.

Morphology.—Bacillus subtilis is from 4 to 8 μ by about 0.7 μ, has rounded ends, is slightly motile, carries spores near, but not exactly, in the middle, and is arranged singly, in pairs and in long filaments.

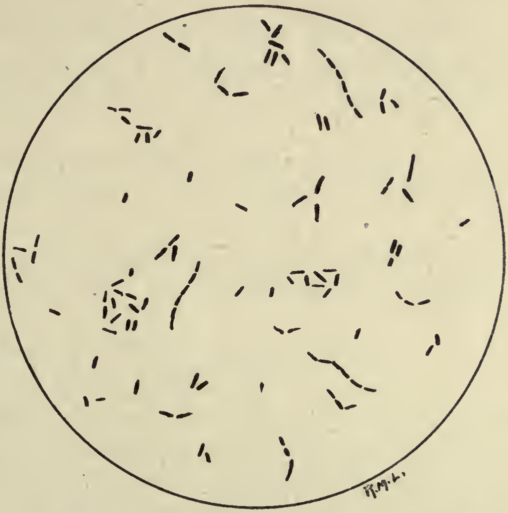

FIG. 27.—BACILLUS SUBTILIS. STAINED BY GRAM'S METHOD.
(4× eyepiece and 1/12 oil immersion objective.)

Staining.—It stains readily with all the usual anilin stains and is Gram positive.

Growth.—Bacillus subtilis is aerobic, and, to a degree, anaerobic. It grows well between 20°C. and 37°C. on all culture media, forming a whitish sediment in fluid media and forming on solid media round, irregular-edged, whitish colonies which tend to coalesce. Gelatin is liquefied.

To insure sterilization by hot air a temperature of 150°C. must be maintained for several hours.

Boiling for 1 hour, or 20 minutes, in an autoclave at 15 pounds pressure kills bacillus subtilis spores.

Four or five daily exposures at 80°C. for 10 minutes is sometimes required when sterilization is to be accomplished by the fractional or intermittent method.

When a pure culture of bacillus subtilis is desired for study and none is at hand, some hay dust is placed in sterile water, gradually heated to the boiling point and then boiled for 20 minutes.

If tubes of agar are planted with several loopsful of this decoction—immediately before heating, when it reaches 60°C., 80°C. and 100°C., and again when it has boiled for 1 minute, 2 minutes, 5 minutes, 10 minutes, 15 minutes and 20 minutes—a pure culture of subtilis will be obtained and a study of the culture made before and after exposure to different degrees of heating will show the variations in the heat-resisting power and different bacteria and spores.

CHAPTER XXXVIII

BACILLUS PRODIGIOSUS

Bacillus prodigiosus occurs in soil, dust and water, and upon exposed foodstuffs, especially those containing starch.

Morphology.—Bacillus prodigiosus is smaller than the typhoid bacillus, but the difference is not sufficient to permit differentiation. It is motile and possesses from six to eight lateral flagella. It is arranged singly, in pairs, short filaments and in irregular groups.

Staining.—It stains readily with all the anilin dyes and is Gram negative.

Growth.—Bacillus prodigiosus is aerobic and facultative anaerobic. Under aerobic conditions it produces a red pigment; this does not occur in an anaerobic state. It grows best between 20°C. and 25°C.; above 37.5°C. pigment is not formed.

Bouillon becomes cloudy in 24 to 48 hours; under favorable circumstances pigment forms and gives the medium a reddish tint; after several days a sediment forms.

Agar.—Growth appears in 18 to 24 hours, as round, moist, glistening, whitish, pin-head-sized colonies, which later coalesce and cover the surface with a red pellicle.

Gelatin is liquefied and a reddish sediment forms.

Milk is acidulated and coagulated.

Potato is the best medium upon which to observe pigment formation. Growth covers the entire surface in 24 to 36 hours. At first it is bright red; as the culture ages the pigment darkens until it becomes brown.

Glucose is not acidulated, but occasionally gas is formed. Indol production is variable. Bacillus prodigiosus does not form spores.

Resistance.—Bacillus prodigiosus is sterilized by an exposure of ½ hour to 150°C. dry heat, or ½ hour at 80°C. moist heat; boiling kills it immediately. It is slightly more resistant to chemical germicides than the typhoid bacillus.

Toxin.—Very old cultures are said to contain toxin.

Agglutinins.—Specific agglutinins are present in the serum of immunized animals.

Pathogenesis.—Bacillus prodigiosus is a saprophyte, in old cultures it is occasionally slightly pathogenic. This organism is of interest to physicians because it is frequently present in dirty foods and may alter them in a deleterious manner; also it is used in preparing Coley's fluid, an agent used in the treatment of certain cases of sarcoma.

CHAPTER XXXIX

BACILLUS PESTIS

Bacillus pestis is present in the lymphatic glands and in the blood of infected animals and man; in the sputum of those suffering from the pneumonic form of the disease, in the carcasses of those dead of plague and in fleas which have fed upon infected rats. It has been found several times in the soil of districts where plague is endemic.

Morphology.—Bacillus pestis is subject to marked variations in size and shape. Bacilli removed from the lymphatic glands of those suffering with plague are about 1 μ to 2 μ have decidedly rounded ends, stain deeply at each end and show a clear band in the middle. They are arranged singly. Young (24 to 48 hours) cultures in bouillon, agar and salt-agar have a different appearance; bacilli from bouillon stain solidly, are about 1 μ by 2 μ and are arranged in pairs, end to end, and in filaments, as well as singly. Those from agar and salt-agar have the same appearance, but do not show filaments as in bouillon.

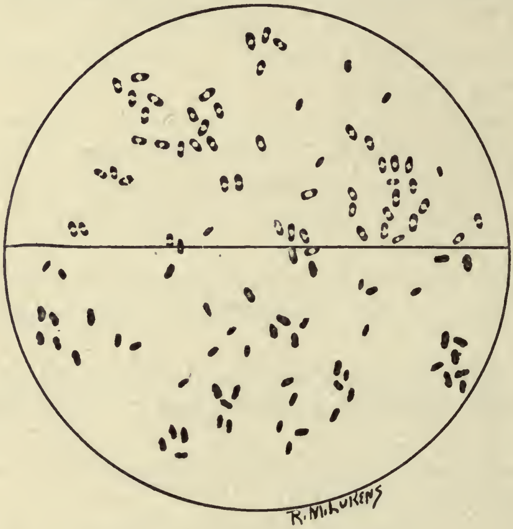

FIG. 28.—BACILLUS PESTIS.
Upper half of field shows polar staining. Lower half of field shows solid staining.
(4 × eyepiece and 1/12 oil immersion objective.)

Old agar and salt-agar cultures (5 to 6 days old) show marked involution forms, bacilli 3 or 4 μ long and 1 μ wide showing polar staining, and larger bacilli which stain solidly and are curved or club-shaped. Similar involution forms may be found in tissue. Bacillus pestis is not motile.

Staining.—Bacillus pestis stains with all the basic anilin dyes and is Gram negative.

Growth.—Bacillus pestis is an aerobe and grows best at temperatures between 30°C. and 38°C.

Bouillon growth appears as a whitish sediment, which sticks to the sides or falls to the bottom of the container. The medium usually remains clear, occasionally it becomes turbid; slight pellicle formation may occur.

Bouillon to which sufficient sterile oil or butter has been added to form globules on the surface, if kept at rest during incubation, has a stalactite formation if bacteria develop. Around each globule these stalactites are whitish and when the medium is agitated they fall to the bottom.

Agar, after incubation at 37°C. for 24 hours, shows small, round, irregular-edged, transparent whitish colonies. Subcultures show more abundant growth than those made from tissue.

Salt-agar is especially valuable to demonstrate involution forms.

Gelatin surface cultures show round yellowish colonies.

Gelatin stab cultures show a fine thread-like whitish growth along the stab with a yellowish growth at the surface. Gelatin is not liquefied.

Blood serum growth appears as on agar.

Milk is not coagulated; there may be slight acidulation.

Potato shows slight, if any growth without any distinctive feature.

Bacillus pestis forms acid, but no gas in glucose; there is neither acid nor gas production in lactose and saccharose. Spores are not formed. Indol is not formed.

Resistance.—Exposure to direct sunlight will kill the bacillus pestis in several hours. In dried pus it remains alive for weeks. It has been found alive in soil, protected from light, after several months and in water after 1 month. It remains alive and virulent in the bodies of those dead of plague for weeks; when putrefaction occurs bacillus pestis is said to disappear in 15 to 30 days. Five per cent. carbolic acid solution kills it in 10 minutes, so does 1:1000 bichloride. Hot-air sterilization requires an exposure of ½ hour at 150°C.; in a moist state 60°C. for 1 hour. Boiling kills it instantly.

Toxin.—Bacillus pestis produces an intracellular toxin.

Agglutinins.—The occurrence of agglutinins and precipitins in the blood of infected and immunized persons and animals is irregular and slight.

Pathogenesis.—Plague is a disease said to occur in many animals other than man and rats; among them may be mentioned squirrels and guinea-pigs, rabbits, cats, chickens and monkeys.

Much the most common and important is the occurrence of the disease in rats and man. The disease usually prevails in rats of a district shortly before the occurrence of an epidemic among the people; this association is almost a rule and the agent of transmission is the flea. Fleas which bite stricken rats imbibe the bacilli and implant them upon the skin of people whom they bite. Some believe the flies and mosquitoes may play a minor part in the dissemination of plague.

Two forms of the disease occur in man: the bubonic, distinguished by the enlargement of many or all of the superficial lymph glands, with or without, usually without, involvement of the lungs, and the pneumonic form, distinguished by the occurrence of pneumonia with slight, or no enlargement of superficial lymph glands.

In both the bubonic and pneumonic forms of the disease, bacteremia is said to occur; in both bacilli are present in the lymph glands and may be in the urine.

If the bubonic form is complicated with pneumonia bacilli may occur in the sputum. In the pneumonic form bacilli are regularly present in the sputum in large numbers, from the onset throughout the disease.

Diagnosis.—Microscopic examination of the sputum in pneumonic cases discloses the cause.

For the diagnosis of suspected and bubonic cases lymph glands are searched for, massaged and some of their contents removed with a sterile syringe and needle. A portion of the material so obtained is planted on salt-agar, in bouillon containing oil and in MacConkey's bile-salt medium; about 1/10 to 1/4 cc. is injected subcutaneously into a guinea-pig, smears are made, fixed and stained with carbolfuchsin, others with methylene blue and still others by Gram's method.

The discovery of a polar staining cocco bacillus in the smears, involution forms on salt-agar after 24 hours at 37°C., death of the inoculated animal and recovery from its body of organisms identical with those removed from the gland establishes the diagnosis.

When examining rats, in addition to making blood cultures, and subculturing these on glucose, lactose, saccharose and salt-agar, careful examination of the spleen and liver should be made to detect characteristic changes, produced by plague.

CHAPTER XL

BACILLUS MALLEI

(GLANDERS BACILLUS)

The bacillus mallei is present in the nasal discharge and saliva of many of those suffering from the disease, also in the exudate from open skin lesions. From such sources the dust and soil of stables and horse troughs are at times polluted.

Glanders is a disease of horses, asses and mules, occasionally carnivora fed the meat of animals dead of the disease develop glanders, and the disease occasionally afflicts man.

Infection with glanders is confined almost entirely to persons in frequent contact with horses.

Morphology.—Bacillus mallei occurs both as straight and slightly curved, non-motile rods, 3 μ to 4 μ long, 0.50 μ to 0.75 μ wide. having rounded ends, and arranged singly in irregular masses. Old cultures show larger, club-shaped, granular forms, long (apparently branched) filamentous forms, and chains of small coccoid forms. Bacillus mallei does not form spores.

Staining.—This bacillus stains readily with all the common stains and is Gram negative. It can be decolorized, after staining, by washing with water, much easier than other bacteria. As a rule, staining is not uniform, many organisms showing an oval unstained central portion, falsely suggesting the presence of spores.

Growth.—Occurs from 23°C. to 41°C., best at 37°C. Bacillus mallei is aerobic and only slightly facultative anaerobic.

Bouillon incubated at 37°C. becomes cloudy in 24 hours, later a tough, mucoid, white sediment appears and in several days a white pellicle sometimes covers the surface.

Agar incubated at 37°C. shows surface growth in 20 to 24 hours, at first a white, transparent streak, which spreads, thickens and becomes opaque as growth continues.

Glycerin agar favors a more abundant growth, the surface usually being covered with a film having the same appearance as observed on plain agar.

Gelatin.—Scant, almost invisible growth occurs upon or in gelatin after several days, it is not liquefied.

Potato, neutral or nearly neutral in reaction is an important medium in the study of the glanders bacillus. In 48 hours, incubated at 37°C., a characteristic, moist, thick, yellowish solid growth develops. As the culture ages it turns brown and the potato adjacent to it becomes black.

Milk is acidulated and in 1 to 2 weeks slowly coagulates.

Resistance.—In moist state bacillus mallei is killed in 1 hour at 80°C., in the Arnold steam sterilizer and autoclave it is destroyed in 1 or 2 minutes. Thorough drying rapidly attenuates and soon kills it. In the hot-air sterilizer it is killed within 1 hour at 120°C. At room temperature, 5 per cent. phenol solution kills it in less than 15 minutes; 1:1000 bichloride of mercury in less than 45 minutes. Complete exposure to direct sunlight will kill it in several days. This bacillus has been found alive and virulent after remaining 60 days in a water trough.

Toxin.—Bacillus mallei produces an intracellular toxin, mallein, obtained the same as tuberculin, and employed in the diagnosis of glanders in animals, the same as tuberculin is employed in the diagnosis of tuberculosis in cattle; with this exception—at present mallein is usually dropped on the eye, not injected subcutaneously. There is no extracellular toxin production.

Pathogenesis.—Infection in man is usually through an abrasion of the skin, a nodule developing at the atrium of infection is rapidly surrounded by a zone of intense inflammation. High fever, severe malaise, a general papular eruption that becomes pustular frequently occurs in severe acute cases which are often fatal in 2 or 3 weeks.

Less acute cases and those terminating in recovery may not develop a generalized eruption. Infections running a chronic course in man are marked by enlargement of the lymphatic glands and vessels and are often fatal.

Rabbits and guinea-pigs are susceptible to inoculation.

Diagnosis.—Laboratory diagnosis is based on microscopical and cultural studies of the exudate from open lesions, or of fluid aspirated from enlarged glands, or excised glands emulsified with normal salt solution, and the result of guinea-pig inoculation.

The exudate from open lesions usually contains organisms in addition to the Glanders bacillus, and should be thoroughly cleansed and covered with a moist aseptic dressing for several hours before specimen is removed for examination. Suspected material is planted on potato medium.

When inoculation test is to be made, exudates and superficial tissue apt to be contaminated with other organisms are injected subcutaneously into a guinea-pig. When nodules develop or the pig dies, under strict aseptic precautions, a nodule is excised, placed in a glass mortar with several times its volume of normal salt solution and emulsified. One or 2 cc. of this emulsion is injected into the peritoneal cavity of a male guinea-pig.

Subcutaneous nodules removed from patients are emulsified and injected directly into the peritoneal cavity of a male guinea-pig.

When injected this way, in pure culture, into male guinea-pigs, bacillus mallei, frequently, but not always, causes a severe orchitis with enlargement and suppuration.

CHAPTER XLI

SPIROCHÆTA OBERMEYERI

(SPIROCHÆTA, OF RELAPSING FEVER)

Spirochæta Obermeyeri occurs in the blood of those suffering with relapsing fever and in certain lice, bugs and ticks that have fed upon infected persons.

Morphology.—Spirochæta Obermeyeri show marked variations in length; the average dimensions are 20 μ long by 1 μ wide. It is spiral, the curves being of uneven lengths and depth. It is actively motile and progresses with a screw-like motion. The ends are pointed and it is said to possess terminal flagella.

Staining.—Same as for treponema pallidum (see page 150).

Growth.—Cultivation on artificial media is difficult; most attempts result in failure and it is not necessary for diagnostic purposes.

Pathogenesis.—Inoculation with blood taken from relapsing fever patients has produced the disease in monkeys and rats. Rabbits and guinea-pigs are immune. Spirochæta Obermeyeri has been found alive and infectious in ticks and leeches several days after they fed upon infected persons. When the body louse imbibes this spirillum it may pass it through heredity to the next generation.

Diagnosis.—In suspected cases blood should be obtained from the finger during the febrile period. Several thin films should be made on clean slides or cover glasses; these should be dried at room temperature or placed in an incubator, not flamed. At the same time a drop of blood should be examined in the fresh state. Place it on a clean slide, gently drop a clean cover upon it and immediately examine with an oil immersion lens.

The organisms, most of them five or ten times as long as the diameter of a red blood cell, are observed moving about with great rapidity, by a screw-like and also undulatory motion, often pushing red cells aside.

During the febrile period of the disease spirochæta Obermeyeri retreats from the peripheral blood and examination at this time is not advisable.

Differentiation of the closely related spirochæta can be made only by agglutination tests with the serum of immunized rats.

CHAPTER XLII

TREPONEMA PALLIDUM

Treponema pallidum is present in the ulcers and skin lesions of syphilis, most numerous in the primary and secondary period of the disease, scant later in the infection. In small numbers they are present in the blood at times during the disease and present in the enlarged glands and affected organs.

Morphology.—Treponema pallidum is variable in length, averaging about 6 μ long and 0.25 μ in diameter. It is spiral, showing from five to twenty curves, which are fairly uniform in depth and length; they are deepest at the center and gradually become less marked toward the ends, just like a corkscrew. The ends are pointed and there is said to be a single terminal flagellum. Treponema pallidum is actively motile and progresses with a screw-like motion; lateral motion is less marked. Reproduction occurs by both longitudinal and transverse division.

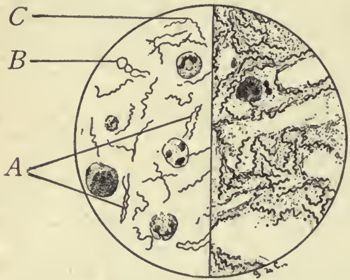

FIG. 29.—TREPONEMA PALLIDUM. (*Coplin.*)

Left half, Giemsa stain, spread from congenital syphilis; right half, Levaditi stain of section of lung, congenital syphilis; this (silver method) makes the organisms appear much broader. *A*, Pairs united at one end. *B*, Twisted pairs. *C*, Double length, apparently dividing form.

Staining.—Ordinary bacterial stains fail to or only poorly stain treponema pallidum. The best method of staining smears is the following:

GIEMSA'S METHOD

1. Make a thin, even smear, dry in air without heating.
2. Fix in absolute alcohol for ½ hour.
3. Apply the following stain for 1 hour:

Giemsa's solution (Grübler), 1 part.
1 per cent. aqueous sol. sodium carbonate, 1 part.
Distilled water, 1 part.

After staining, wash with distilled water, dry in air and mount.

The stain must be freshly prepared just before using.

For the staining of treponema in tissue the following is most satisfactory:

LEVADITI'S METHOD

1. Cut the fresh tissue into small, thin pieces.
2. Fix in 10 per cent. formalin for 24 hours.
3. Harden in absolute alcohol for 24 hours.
4. Wash in distilled water.
5. Place in following solution, freshly prepared:

2 per cent. aqueous solution of silver nitrate, 90 cc.

Keep in this bath for 3 days at body temperature, excluding light and changing the solution daily.

6. Place in the following solution for 24 hours at room temperature:
   5 per cent. solution of formaldehyde, 50 cc.
   Pyrogallic acid, 1 Gm.
7. Wash in distilled water.
8. Dehydrate in absolute alcohol.
9. Clear in xylol.
10. Imbed in paraffin.
11. Section and mount.

Treponema appear black.

Growth.—Cultivation of treponema pallidum on artificial media is difficult and time-consuming; most attempts are futile and as yet this method of study is not practical in diagnosis.

Material taken from chancres and other open lesions containing treponema also contains numerous other organisms and isolation of the treponema from these is effected by inoculating the material into a rabbit's testicle.

When the contaminating organisms die out and the treponema multiply, media planted with an emulsion of such a testicle yield pure cultures.

The treponema pallidum is an obligate anaerobic and has only been propagated in ascitic broth and ascitic agar containing a piece of sterile rabbit testicle.

Stab cultures in deep tubes of agar, incubated at 37°C., after several days, show growth near the bottom of the tube which first appears as fine white lines radiating from the stab.

The character of lesion that develops following inoculation of a rabbit depends upon the tissue in which the treponema are deposited. If deposited in the subcutaneous tissue of the scrotum a chancre usually develops. If deposited deep in the testicular tissue gumma develop. The production of gumma is desired when rabbits are inoculated as a primary step to procure pure cultures, because the gumma are most apt to contain spirochæta not associated with other organisms.

Resistance.—Treponema pallidum is more susceptible to mercury and salvarsan than to other chemical germicides. It does not form spores and probably is destroyed by short exposure to moderate degrees of heat.

The time it can remain alive and infectious on linen, glass, soap and other objects, subject to contamination is undetermined; that it does survive for short periods at least on such objects has been proved by infections contracted through contact with them.

Pathogenesis.—Treponema pallidum occurs in man only. Conditions similar to syphilis can be produced in monkeys and rabbits by inoculation. Syphilis, like some other diseases, produces a hypersusceptibility of the skin to irritants in general and especially to the infecting organism. In syphilis this phenomenon is most marked in the tertiary period. Noguchi has attempted to base a diagnostic test on this fact—the test is not generally available on account of the difficulty of obtaining pure cultures of treponema pallidum, the almost impossible feat of differentiating similar spirochæta from the treponema, and the difficulty of keeping pure cultures from contamination. Toward the end of the primary or early in the secondary stage of syphilis a number of properties are demonstrable in the blood, which are not, practically speaking, found in the blood of healthy or diseased persons free of syphilis. By far the most important of these, from a diagnostic consideration, are amboceptors or complement fixing bodies. These are nearly always demonstrable when the disease is active and are also demonstrable in the majority of latent cases. To detect the presence or absence of these antibodies the Wassermann test is employed.

Diagnosis.—In the first stage of the disease ulcers and adjacent enlarged glands should be examined for treponema and a Wassermann test made. A negative Wassermann at this time has no significance.

In the second stage of the disease a Wassermann test should be made, and, if desired, papules, ulcers and enlarged glands adjacent to them may be examined for treponema.

At this time nearly all cases of syphilis give a positive Wassermann; a negative is suggestive of non-syphilitic nature of the malady.

In the third stage of the disease the Wassermann test should be performed as in the primary and secondary stages, and if negative the spinal fluid should then be examined, because at this time a positive reaction may be obtained with the spinal fluid, even though the blood is negative.

EXAMINATION OF ULCERS FOR TREPONEMA PALLIDUM

Wash the ulcer free of extraneous matter with sterile normal salt solution. Avoid bleeding and scrape some material from the ulcer. Place a small portion of the scrapings in a drop of salt solution on a slide, gently drop a cover glass on it and lute with vaseline or paraffin. This should be examined with the dark-field illuminator, or if that is not available, with a 1/12 oil immersion objective and an 18 ocular.

When the scrapings are obtained several slides should be prepared by spreading thin, even films, drying them in air or in an incubator at 37°C., fixing in alcohol and staining by Giemsa's method.

If, in such preparations, an organism is observed that appears to be treponema pallidum, one should remember that a number of organisms, microscopically indistinguishable from treponema pallidum, may be found in superficial ulcers.

Failure to find treponema pallidum in scrapings from ulcers and in fluid from papules and glands does not indicate the absence of syphilis, as the organisms are irregularly distributed and often scant in the material taken for examination.

EXAMINATION OF GLANDS

Enlarged glands should be searched for; if found, they are massaged, the overlying skin asepticized and some of the glandular contents removed with a sterile syringe for microscopic examination. When material so obtained contains motile organisms having the morphology of treponema pallidum, it is strong, one might say conclusive, evidence of syphilis, as contamination is practically precluded.

EXAMINATION OF PAPULES

The admixture of blood with material to be examined is avoided by pinching up the papule with a hemostat, nicking the skin with a knife and obtaining the serum with a capillary tube.

Experience has shown there is great irregularity in the distribution of treponema in tissues, so that some sections of an organ will present few or no treponema while other sections of the same organ present many in each microscopic field.

The application of germicidal washes and dressings, especially those containing mercury, to open syphilitic lesions, causes a disappearance of treponema and attempts to find organisms in scrapings and fluid from such lesions are usually futile until after such washings and dressings have been discontinued for at least 12, better 24 hours.

Subcutaneous and intracutaneous injections of cocain, and other methods of producing local anesthesia, usually decreases the number of treponema that may be found in syphilitic lesions or causes the treponema to totally disappear, hence no attempt at producing local anesthesia should precede removal of fluid or scrapings from ulcers, papules or lymphatic enlargements for examination with the dark field microscope or microscopic examination after staining and for the same reason no anesthetic should be applied prior to excision of tissue to be examined for treponema.

THE WASSERMANN TEST

1. Under aseptic precautions at least 5 cc. of blood are obtained from a prominent vein at the elbow with a sterile syringe; the blood is immediately transferred to a sterile tube and placed in an ice box until serum separates from the clot.

2. The serum is collected and heated to 55°C. for ½ hour in a water bath.

3. The patient's serum is mixed with fresh guinea-pig serum and liver extract and incubated at 37° for 1 hour.

4. After incubating for an hour red blood corpuscles and serum of a rabbit, immunized against them, is placed in the tube, which is again incubated for an hour.

If hemolysis occurs the reaction is negative (see page 241).

CHAPTER XLIII

SPIROCHÆTA PERTENUIS
SPIROCHÆTA REFRINGENS AND SPIROCHÆTA DENTIUM

Spirochæta pertenuis is the cause of a non-venereal tropical skin disease, called "frambœsia tropica," or "yaws." Spirochæta pertenuis is regularly found in the papules of the diseased before treatment. It has the same size, shape and motility as the treponema pallidum and when subjected to various methods of staining reacts just as the treponema does except that as a rule spirochæta pertenuis stains more readily. Patients infected with yaws have a positive Wassermann reaction just the same as syphilitics and yaws promptly answers to salvarsan and mercury.

Some observers believe there is a close relationship between the treponema pallidum and spirochæta pertenuis.

Diagnosis is made by pinching one or several of the skin lesions with a hemostat, nicking it with a scalpel so as to obtain serum and not blood. The fluid that exudes is collected in a capillary tube, smeared on cover glasses, dried, fixed in absolute alcohol for 15 minutes and stained with equal parts of Giemsa's solution and water for 20 minutes.

The large number of distinctly stained spirochæta observed in every cover-glass preparation, the abundance of these organisms in every lesion, of a fully developed case of yaws, untreated, serves to establish the diagnosis and differentiate it from syphilis—the lesions of which do not so uniformly yield fluid containing demonstrable treponema and almost never show so many organisms.

Spirochæta dentium is a saprophyte present in the mouth, particularly between the teeth. It is microscopically indistinguishable from the treponema pallidum.

Spirochæta refringens has been found in ulcers of the skin, in smegma and in the mouth. Usually it is larger and more easily stained than the treponema pallidum, yet microscopic differentiation is at times impossible and often questionable.

CHAPTER XLIV

THE HIGHER BACTERIA

Between the lower forms of bacteria, the unicellular rods, spheres and spirals, which reproduce by fission only, and the true molds which have differentiated parts, comparable to the roots, trunk and fruit of trees, there are intermediate forms difficult to classify satisfactorily. Three groups in this class are of interest to the medical bacteriologist: leptothrix, cladothrix and streptothrix; sometimes referred to collectively as *trichomycetes*.

LEPTOTHRICES

Leptothrices have been found in inflammatory lesions of the mouth; they are frequently observed in saliva and sputum from healthy persons, more often when there are decaying and ill-cared-for teeth.

Morphology.—They are rod-shaped and vary in length from 30 to 150 μ and in width from 0.5 to 2 μ; usually they are seen about 1 μ wide and 50 μ long. Some are straight, others curved or twisted, and they are arranged singly, in filaments and in clumps. Some forms are segmented, others are not.

Staining.—Leptothrices stain readily with the usual aniline dyes.

Growth.—Attempts to cultivate on artificial media have been unsuccessful.

Pathogenesis.—The leptothrices, in all probability, are non-pathogenic and occur in the mouth, sputum or saliva as saprophytes.

CLADOTHRICES

Cladothrices are distinguished from leptothrices and streptothrices by false branching. They are, therefore, distinguished from leptothrices, which do not show branching. Whether or not reported cases of cladothrix infection were such, is a mooted question, as differentiation between true and false branching is difficult to determine. In any case, cladothrix infections are not common if they do occur and clinically are similar to streptothrix infections.

STREPTOTHRICES

Streptothrices are distinguished by true branching, septate filaments and reproduction by conidia, which form in rows.

Long filaments may be observed singly and intertwined. Some appear chiefly as short, stout rods. Frequently both short and long filaments terminate in a club-shaped swelling.

STREPTOTHRIX ACTINOMYCOSIS

Streptothrix actinomycosis occurs upon grain and pasture land contaminated with the discharges from open lesions of cattle, and in the pus and sinus discharges of the disease, also in sputum when the lungs are involved.

Morphology.—Pus or fluid discharging from an actinomycotic lesion shows hard, brittle, irregular, pin-point-sized, yellowish and whitish granules. Place one of these granules on a slide, in a drop of glycerin and crush it with a cover glass. Microscopic examination will reveal rosette-like masses. The dense centers of these are composed of interwoven filaments, and the outer zone of club-shaped bodies arranged in ray form. Here and there, isolated clubs and filaments are observed, occasionally a filament terminating in a club-shaped body. The filaments vary in length from 5 to 15 μ, the clubs are about 25 to 10 μ. The addition of a drop of 20 per cent. potassium hydrate, or a drop of Gram's solution, makes them appear more distinctly. These granules are not always present. Early in the disease, when resistance to infection is slight, the pus may only contain filaments. The filaments are long and short and show branching.

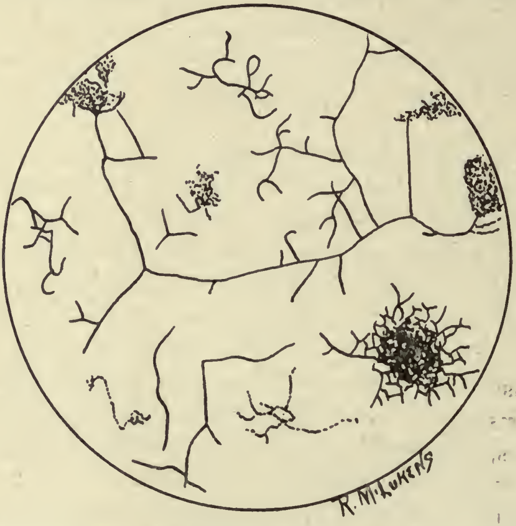

Fig. 30.—Actinomyces, Showing Branching of the Filaments. Stained with Fuchsin. (4 × eyepiece and 1/12 oil immersion objective.)

They stain with all the usual anilin dyes and are Gram positive and are acid-fast (to a lesser degree than tubercle bacilli), but are not alcohol-fast.

Organisms removed from culture media appear differently. Young cultures show fine, branching, non-septate filaments, some of which are very long. Older cultures, that have developed aerial hyphæ, show short, thick, straight rods; some have aerial hyphæ branching from them and these aerial hyphæ terminate in a row of round, irregular-sized conidia; club-shaped forms are not observed. Cultures do not show rosette forms. Involution forms, somewhat similar to, but larger, than involution forms of diphtheria bacilli, are observed in very old cultures.

Growth.—Streptothrix actinomyces is equally adapted to aerobic and anaerobic cultivation. Growth occurs at temperatures between 20°C. and 50°C., best at 37°C.

Bouillon shows growth after 5 or 6 days' incubation at 37°C. A whitish flocculent sediment and a whitish surface pellicle forms without clouding the medium.

Glycerin bouillon cultures have the same appearance as plain bouillon cultures.

Gelatin.—Small, round, white, glistening colonies appear after 5 or 6 days. They continue to increase in size for several weeks and become yellowish in the center. Growth is scant on this medium and it is liquefied very slowly.

Glycerin agar shows growth in 2 or 3 days. At first small, wrinkled, dry, white colonies appear; these coalesce and form a dry, wrinkled pellicle, which may be white, yellowish-white or yellow; aerial hyphæ appear as irregular projections from the surface.

Potato.—Growth first appears similar to that on agar; the pellicle, as it ages, is first yellow, then brown and finally black.

Milk is not coagulated.

Resistance.—Streptothrix actinomyces is more resistant to most germicidal agents than the tubercle bacillus.

Toxin.—Streptothrix actinomyces produces an intracellular toxin.

Pathogenesis.—Actinomycosis is most frequently seen in cattle, the site of infection in most cases being the lower jaw. This results from eating grain or grass covered with actinomyces. When taken into the mouth an abrasion of the mucosa or a necrotic tooth offers a favorable site for infection; the organism enters and the disease progresses. Its course is chronic and gradually a large, hard swelling forms, a condition commonly known as "lumpy jaw;" eventually suppuration occurs, then spontaneous evacuation. Pus oozes from the opening.

Infection does not always involve the jaw. It may be localized in the tongue, skin, peritoneum, pleura or lungs and septicemia or generalized infections have been observed.

When the lungs, pleura or peritoneum are involved the condition simulates tuberculosis. In man conditions following infection are similar to those observed in cattle. The disease affects, most frequently, those engaged in agricultural occupations and the care of cattle.

Horses, sheep, and hogs are sometimes infected and the disease can be produced in rabbits and guinea-pigs by inoculation.

Usually secondary infection occurs; staphylococci, streptococci and other organisms are nearly always present in the pus discharged from actinomycotic lesions.

In the sputum from pulmonary cases tubercle bacilli may be found, together with streptothrix actinomycosis, and should be sought for in such cases.

Diagnosis.—Fluid or pus from suspected cases should be carefully examined for yellowish, hard granules. If any are found they should be crushed between a slide and cover glass and examined for rosette-like masses of club-shaped bodies and for filaments. If granules are not discovered, then the pus is smeared on a slide and examined for rosettes. The suspected material must be examined as soon after removal from the lesion as possible.

The finding of typical rosettes, clubs and filaments establishes the diagnosis.

When, for more detailed study, cultures are desired the actinomyces must be isolated from other organisms present in the pus. The technique for this is as follows:

Lift a dozen or more of the granules from the pus and place on gelatin plates; incubate these plates at 37°C. for 2 days; most of the granules will be surrounded with colonies of contaminating bacteria; search for granules free from other bacteria and remove these with a sterile loop and plant on slant tubes of glycerin agar and tubes of Loeffler's blood serum; incubate these tubes at 37°C. Many tubes should be planted, as some will show no growth and others will contain organisms other than actinomyces; tubes containing only streptothrix actinomyces will show growth in 4 to 6 days.

STREPTOTHRIX MADURÆ

Streptothrix maduræ is found in the lesions and pus and exudate of a disease known as "madura foot."

Morphology.—Granules removed from lesions, exudate or pus, consist of interlaced, branching filaments. Organisms removed from cultures have the same appearance; in addition they show spores arranged singly, in pairs, and irregular masses.

Staining readily occurs with the usual anilin dyes. It is Gram positive.

Growth.—Streptothrix maduræ is an obligate aerobe and grows best at 37°C. In bouillon, gelatin, agar, egg and serum growth is scant when it takes place at all.

Glucose Glycerin Agar.—After several days, round, smooth, elevated, yellowish-white colonies appear; as they age they increase in size, become pink and later red. Umbilication may occur or the colonies may coalesce.

Potato.—Warty colonies develop on this medium; at first white, they gradually change to deep red.

Milk is not coagulated.

Hay Infusion.—Flocculi form after several days; some precipitate and some float. Those exposed to the oxygen after several weeks turn from white to pink. The medium is not clouded.

Pathogenesis.—Streptothrix maduræ causes a chronic productive inflammatory condition of the foot; associated nodular swellings give a warty appearance to the surface. After a time suppuration occurs, followed by spontaneous rupture and sinus formation. The exudate or pus which oozes from the sinus contains small white, yellow or black granules similar to those found in actinomycosis.

Rarely the hand is affected. The disease has been observed in man only. Animals are immune.

Diagnosis.—In suspected cases the pus is inspected for granules, and these are stained and examined microscopically.

Cultures are obtained by asepticizing the skin over a nodule, making an incision and removing some fluid from the nodule with a sterile tube and transferring it to hay infusion or glycerin glucose agar.

ACHORION SCHONLEINI

Achorion schonleini is present in the hairs and scabs of tinea favosa or favus.

Morphology.—When one of the crusts containing a hair is removed from the affected area and examined microscopically, branched chains of mycelial spores are observed within the hair. These spores are round, or nearly so, and measure from 3 to 6 μ in diameter. Here and there along the hair mycelial filaments occur and in the crusts or epithelium adjacent to them masses of tangled filaments occur. The filaments are delicate and vary in length; some show several branches and others do not branch.

Growth.—Achorion schonleini is aerobic and grows at temperatures between 15°C. and 38°C., best at 33°C.

Neutral Glycerin Bouillon.—A dark yellow surface pellicle form on this medium.

Neutral Glycerin Agar.—A dark yellow, dry, wrinkled, umbilicated film develops.

Potato.—Growth first is grayish yellow; then darker, until the potato turns brown. It covers the surface with a thick film.

Pathogenesis.—Achorion schonleini is the cause of favus.

"Tinea favosa, or favus, is a contagious vegetable parasitic disease of the skin, characterized by pin-head to pea-sized, friable, umbilicated, cup-shaped, yellow crusts, each usually perforated by a hair. It is usually met with upon the scalp, but may occur upon any part of the integument" (Stelwagon).

Achorion schonleini, or a closely related organism, causes favus of dogs and mice.

Diagnosis.—Bacteriological diagnosis is based on microscopic examination of crusts and hairs removed from affected surfaces.

The crusts and hairs are placed in a drop of 20 to 40 per cent. potassium hydrate solution on a slide and a cover glass is dropped upon it. Gently warm the preparation for a few seconds and then examine. When it is desirable to keep preparations for a time, after warming the slide is placed on a cold surface to rapidly cool it and when cool a drop of eosin-tinted glycerin is allowed to run between cover and slide.

TRICHOPHYTON AND MICROSPORON AUDONINI

Tinia trichophytina, or ring worm, is a vegetable parasitic disease which affects various portions of the epithelial surface of the body, chiefly the scalp or bearded region of the trunk. Several triophyta and the microsporon audouini act as exciting causes of the disease.

The same condition has been observed in dogs and other animals.

Diagnosis.—Bacteriological diagnosis is based upon microscopic examination of hairs removed from the center and outer zone of diseased areas.

The hairs are placed in a drop of 20 to 40 per cent. potassium hydrate solution and a cover glass dropped on them. Several slides should be gently warmed and allowed to stand at room temperature for several minutes before examination. Other slides should be heated until steam arises and then placed on a cool surface to rapidly reduce the temperature, then examined. The microscopic appearance of the most common forms is as follows:

Trichophyton megalosporum endothrix. Affected hairs are swollen and filled with spores arranged in chains. These spores are 5 to 6 μ in diameter.

Trichophyton microides ectothrix, spores no larger than tricophyton megalosporon endothrix, are observed both within and surrounding hairs.

Trichophyton megasporon ectothrix, spores from 8 to 15 μ in diameter, are found both within and surrounding the hairs.

Microsporon audouini, affected hairs are covered with irregularly arranged masses of spores. These spores are round and measure from 1 to 3 μ in diameter. They never penetrate the hair.

MICROSPORON FURFUR

Microsporon furfur is the cause of tinea versicolor (pityriasis versicolor), a skin disease showing a yellow macular eruption on the upper part of the trunk.

Diagnosis.—Scrape the lesions with a knife or glass, place the scrapings in a drop of potassium hydrate solution on a slide and cover with a cover glass.

Collections of filaments and spores are observed between the epithelial cells.

The filaments are 3 to 4 μ in diameter, septate and branched.

The spores occur singly and in irregular masses; they are spherical, 3 to 4 μ in diameter and are encapsulated.

CHAPTER XLV

HYPHOMYCETES (Molds)

Molds are very widely distributed in nature, their spores are so nearly ubiquitous that any article containing pabulum, exposed in a dark, moist atmosphere, is soon covered with a growth.

Only a few molds are pathogenic and infection with such organisms is comparatively rare.

From time to time various hyphomycetes, but most frequently the aspergillus, are reported as the cause of pulmonary infection in man, simulating tuberculosis. As the method of staining sputum to disclose tubercle bacilli is not as apt to reveal hyphomycetes as Gram's method, it is probably the best practice to stain specimens of sputum for diagnosis by both methods and in some cases at least to make cultures. Sputum culture by the method of Petroff will disclose hyphomycetes quite as well as tubercle bacilli.

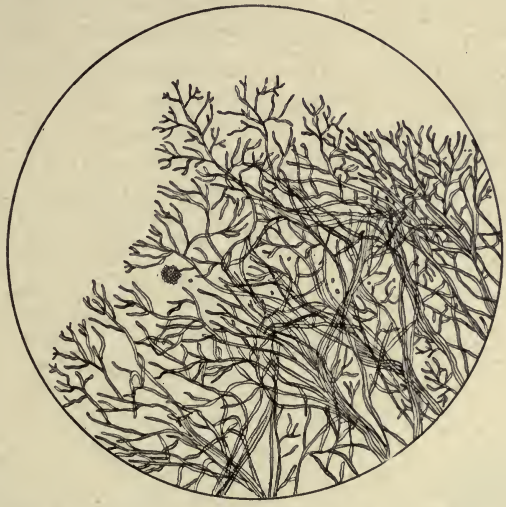

FIG. 31.—MOLD FUNGUS DEVELOPING AS A CONTAMINATION OCCURRING ON AGAR. (8 eyepiece; ⅔ objective.)

Though pathogenic molds will grow on most of the ordinary culture media, Sabouraud's is best: Maltose, 40 Gm.; Peptone, 10 Gm.; Agar, 15 Gm.; Water, 1000 c.c.

Adjust reaction to + 2 and autoclave.

Growth usually does not appear until after 4 or 5 days' incubation, frequently longer.

The hyphæ, mycelia and spores of molds are of such size that their structure and arrangement is discernible when examined unstained with a low-power lens (⅔ to ⅙ objective).

Molds, hyphomycetes or eumycetes are all alike and differ from the algæ in that they (the molds) do not possess chlorophyl.

Pathogenic hyphomycetes belong to one of three classes:

First. **Fungi Imperfecti.**—To this group is relegated all forms not thoroughly studied and those which do not belong to either of the other two classes. Fungi imperfecti includes most of the pathogenic molds.

MOLDS, HYPHOMYCETES OR EUMYCETES

Phycomycetes	Mycomycetes
Hyphæ or **mycelia** show no partitions, entire meshwork of a single organism consisting of one multinucleated cell.	**Hyphæ or Mycelia** show partitions.
Reproduction sexual and asexual, no definite basidium or ascus.	**Reproduction** usually asexual, only definite basidium or ascus.

Mucor	**Aspergillus**	**Penicillium**
Mucor Mucedo	A. Subflavus	P. Galucum
Mucor Pusillus	A. Fumigatus	P. Crustaceum
Mucor Corymbifer	A. Flavus	
	A. Niger	
	A. Concentricus	
	A. Albus	

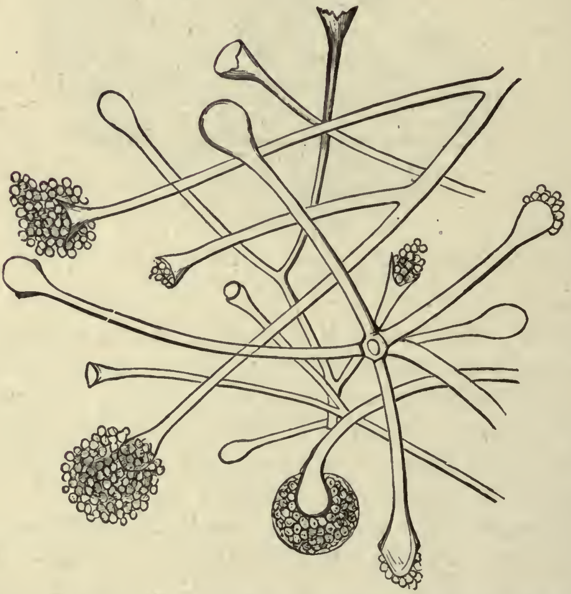

Fig. 32.—Mucor Corymbifer. (*From Plaut after Lichtheim.*)

Asperigillus fumigatus has been found in inflammatory conditions of the upper air passages and in the sputum of persons suffering with pulmonary disease simulating tuberculosis.

Aspergillus fumigatus produces a tubercular-like infection in pigeons, fowl, guinea-pigs and rabbits. Growth on culture media is at first whitish, then green or greenish-blue; later it becomes brown or black.

Penicillium glaucum has been found in chronic inflammatory conditions of the naso-pharynx and Eustachian tube. Growth on culture media is green. This mold is used in the manufacture of certain kinds of cheese.

Mucor mucedo is the mold most commonly observed on foodstuffs; it is whitish and appears like cotton. This organism has been reported as the exciting cause in several cases of tubercular-like infections of the lungs, in which cases it was found in the sputum.

The development of hyphæ, basidia and sporulation of many of the higher forms of bacteria, molds and yeasts can be studied best as follows:

Liquefy a tube of sterile glucose agar, spread a small drop of it in a thin even film on a clean slide with a sterile pipette, and when solid lightly stroke the surface with a platinum loop charged with the material to be studied. Place a cover glass in it and incubate at 37°C. At frequent intervals place on microscope and observe growth with ⅔ and ⅙ objectives and high-power eyepiece 10x to 18x.

Resistance.—The spores of molds are frequently quite as resistant or more resistant to the germicidal influence of heat, light and chemicals than the spores of bacteria. It is a matter of practical importance to know that formaldehyde is less germicidal to molds than to bacterial spores; that molds are especially susceptible to phenol solutions, and very weak solutions of copper sulphate, 1:1000 or less, will prevent their development.

CHAPTER XLVI

SACCHAROMYCETES

(BLASTOMYCETES)

Yeasts are widely distributed in nature, having much the same occurrence as molds. They are the common cause of fermentation and with the exception of a few of the pathogenic species produce an endo-enzyme that splits sugars into alcohol and CO_2. In addition many of them produce other ferments that reduce starches and other complex substances.

Torula or wild yeasts are chiefly of interest as frequent and annoying contaminants.

The cultivated varieties play an important rôle in various industries, especially wine-making, brewing and baking.

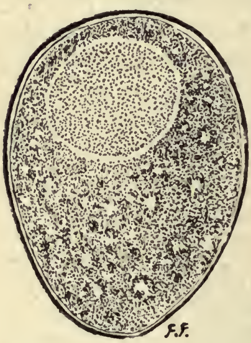

FIG. 33.—YEAST CELL. (*Marshall.*)

As is the case with bacteria and hyphomycetes only a few yeasts are pathogenic.

Morphology.—Saccharomycetes as commonly observed are round or oval unicellular organisms, presenting a distinct cell wall—some species having a double, concentric wall. The protoplasm generally contains numerous small structureless granules, and, less frequently, one or several vacuoles.

They occur singly, in chains and irregular masses. As a whole, individual cells are sufficiently larger than cocci and the spores of molds to be distinguishable from them on sight, but exceptions to this occur: some are as little as 0.5 μ in diameter, they average 10 μ to 40 μ in diameter.

Different species differ in size but individual differences in size in various cultures of a single species under different conditions is great.

Saccharomycetes are non-motile and quite distinctly discernible when not stained. They stain readily with the usual dyes and are Gram positive.

Growth.—There are both aerobic and anaerobic species. Isolated pathogenic species are aerobic and grow well in all the ordinary culture media. Incubated at 37°C., growth becomes apparent in from 24 hours to 5 weeks and continues for a long time.

Young colonies on agar are round, dry, smooth, waxy, white or yellowish, firm and adherent and may attain a diameter of 1 centimeter. Later they become wrinkled, moist and develop aerial hyphæ.

Reported studies of pathogenic species of saccharomyces indicate that many, if not all, under certain conditions form mycelia and aerial hyphæ.

In tissue and actively growing young cultures, all species reproduce by budding, numerous cells showing one or more buds. These buds, which first appear as small globular protrusions from the cell wall, rapidly increase in size and eventually split off, becoming mature cells.

Some, if not all species, under certain conditions, reproduce by sporulation and present cells containing one or more spherical spores—usually four.

Pathogenesis.—Though comparatively rare, infection in man with saccharomycetes is much more frequent than infection with molds. The species infecting man is also pathogenic for dogs, cats and mice, especially the latter. The source of infection is obscure, the onset insidious, the disease essentially chronic and frequently fatal. Reported cases tend to indicate that the nature of such infections usually goes unrecognized.

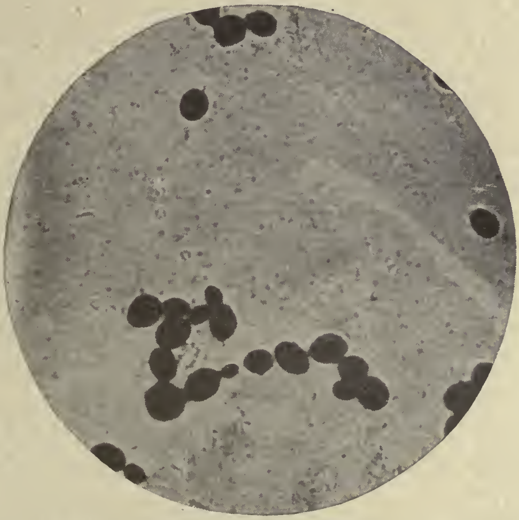

FIG. 34.—YEAST CELLS STAINED WITH FUCHSIN. (×1000.) (*MacNeal.*)

At least three species are pathogenic for man (the investigations of Rabinowitsch indicate more):

Oidium Albicans (Endomyces albicans), which attacks the buccal mucous membrane of ill-cared-for, debilitated infants and, less often, adults. It forms a white growth or pseudomembrane on the affected part and in some cases may cause necrosis of underlying tissue. The condition is known as *Oidiomycosis,* thrush or babies' sore mouth.

Zymonema Gilchristi, which attacks the skin, producing a condition referred to as *Blastomycetic Dermatitis.*

Saccharomyces Tumefacians, which may be a localized infection causing a tumor-like mass, or extensive ulceration, of any part of the body, or a localized pulmonary infection simulating pulmonary tuberculosis or a generalized infection with multiple abscess formation.

Diagnosis.—Microscopic examination of smear made from the affected membrane in oidiomycosis, or made from the serum or pus expressed from skin lesions in blastomycetic dermatitis, or from the sputum in pulmonary blastomycosis, or pus of abscesses, readily discloses the nature of the infection, the large, round and oval, budding cells with a distinct wall being characteristic.

Sections of involved tissue present numerous identical cells.

Saccharomyces Neoformans, a yeast frequently found in fermented fruit and advanced as the cause of cancer by Sanfelice, probably is not pathogenic and has been rejected as the cause of carcinoma.

COCCIDIOIDES IMMITIS

Coccidioides Immitis is the cause of a disease in man—Coccidiosis—which begins as a papular eruption, the papules later coalescing, become pustules, which in turn are followed by ulceration and a purulent discharge.

The infection may remain localized in the skin for months, being confined to the integument of one extremity or involving a larger area. Eventually in most cases the lymphatic glands are attacked and general disseminated infection with clinical signs and pathological changes resembling miliary tuberculosis mark the last stage of its fatal course.

Morphology.—Coccidioides Immitis, in the purulent discharge from lesions, in the giant cells of tubercles found in affected tissue and in material taken from buboes, appears as round, thick-walled, yeast-like cells, 20 μ to 40 μ in diameter, some of which contain spores.

In spreads made from cultures organisms identical to those found in tissue are observed and in addition some show budding and mycelia.

Growth.—Occurs aerobically at room and body temperature on agar, glycerin agar and glucose agar, neutral or acid in reaction.

Pathogenesis.—Subcutaneous inoculation of pus or fluid from lesions and of cultures produces disease in guinea-pigs, rabbits and monkeys similar to that observed in man.

Diagnosis.—Make smears for microscopic examination and cultures on Sabouraud's agar of purulent discharge from skin lesion, or, if the disease has not progressed beyond the papular stage, excise a papule, fix, section, stain and examine serial sections.

When buboes exist, if organisms have not been found in superficial lesions, massage an enlarged gland, aspirate as much as possible of its contents with a syringe and needle and examine the same as pus.

For a detailed description of systemic blastomycosis see Stober, A. M., Archives Int. Med., xiii, No. 4, April, 1914.

CHAPTER XLVII

MONILA

Monila resemble Oidium (endomyces) albicans and are closely related. When first described as a separate genus the distinguishing characteristic of monila was stated to be the absence of ascosporulation.

Based on variations in effect on litmus milk, gelatin, the carbohydrates and agglutination reactions, many species have been recognized in recent years. Castellani has isolated and described the following: monila intestinalis, monila fæcalis, monila insolita and monila tropicalis—all obtained from the saliva, scrapings from the tongue or feces of patients afflicted with sprue—of which disease he does not believe they are the cause. Bahr also has recovered an organism, described as monila albicans, from the tongue and feces of sprue patients and he believes it the cause of their disease.

In scrapings from the tongue, in the saliva and feces of patients having sprue, Ashford has regularly found a monila that in its parasitic state has the same morphology as oidium albicans, that produces mycelia but does not reproduce by ascospores in its vegetative state, that forms gas and acid in glucose, levulose and maltose and does not form gas but acidulates saccharose and galactose media; it does not liquefy gelatin or blood serum and does not coagulate milk but makes it alkaline. This organism is pathogenic for rabbits and is believed by Ashford to be the cause of sprue. He believes the infection may be conveyed to man by bread and states in reference to primary diagnostic cultures, "I consider this medium as specific for monila as Loeffler's blood serum mixture is for the bacillus diphtheriæ"—meaning Sabouraud's glucose agar (4 per cent. glucose) having a reaction of +2.

A reported case of pulmonary moniliasis has been observed in the Johns Hopkins Hospital.

CHAPTER XLVIII

SPOROTRICHUM SCHENKII

Sporotrichum Schenkii occurs in the lesions of sporotrichosis, a chronic infection marked by the occurrence of multiple subcutaneous gumma-like masses which liquefy and discharge material similar to pus. Occasionally other parts are affected, infections of the buccal and pharyngeal mucous membrane, lymphatic glands, bones and synovial membrane having been reported.

Morphology.—Fluid and sections of tissue from lesions show elongated oval, yeast-like cells 3 μ to 6 μ long. Cultures show long delicate, septate, branched filaments with clusters of from 2 to 12 or more brown spherical and oval spores, about 2 μ in diameter at their ends.

Culture growth appears on Sabourand's agar, incubated aerobically at room temperature, in 5 to 10 days. At first white, as it ages the growth darkens, spreads over the entire surface and becomes brown.

Glucose Broth.—After a number of days a white pellicle forms on the surface, the medium remaining clear. In time the pellicle precipitates and a second surface pellicle may form.

Diagnosis.—Massage hard gumma-like masses or glands, if soft ones are not present. With a sterile syringe and large bone needle withdraw the contents, plant as much as possible up to 1 cc. on Sabouraud's agar slants and spread on slides for immediate microscopic examination. Examine both unstained and stained preparations.

When fluid cannot be obtained or contains no organisms, pieces of diseased tissue should be excised, cultured, and also sectioned for microscopic examination.

CHAPTER XLIX

INFECTIOUS DISEASES OF UNKNOWN CAUSATION

Of the diseases afflicting man that have been differentiated about one-half are caused by known organisms.

The method of transmission from person to person, the mode of onset and the course of many other diseases indicate that they too are due to infection, although attempts to isolate and identify the causative organisms have failed. The success of research from time to time is happily reducing this group. It is yet too early for the medical profession to have adequately tested and confirmed the remarkable discoveries of the last 4 years relative to typhus fever, but the evidence strongly suggests that the specific cause of this disease has been disclosed.

Bacillus typhi-exanthematici, first described by Plotz, Olitsky and Baehr, has been obtained in blood cultures from typhus patients, using a 2 per cent. glucose-ascitic-fluid-agar medium. It has also been isolated from lice caught where the disease prevailed and from mice, guinea-pigs and monkeys injected with the blood of typhus patients.

It is a Gram-positive, obligate anaerobic bacillus. The spleen of mice inoculated with this organism is said to show characteristic lesions.

Infection of man has been known for a long time to depend upon transmission by the body louse and hence the prevention of typhus epidemics and the curtailment of existing epidemics is effected by extermination of body lice.

Occasionally before crisis, frequently at the time of the crisis, and in nearly all cases after crisis, the blood serum of typhus patients will cause agglutination of the bacillus typhi-exanthematici, and will cause complement fixation with this antigen.

Opinion differs widely as to the prophylactic and therapeutic value of vaccine. It is worthy of note that Strong has not yet indorsed the conclusions of those who believe in the efficacy of this vaccine.

ACUTE ANTERIOR POLIOMYELITIS

(Epidemic Poliomyelitis)

Acute anterior poliomyelitis is a disease caused by a filterable virus. Emulsions of the brain and cord of persons dead of the disease injected into monkeys and rabbits frequently produce malaise, fever, paralysis and death.

One attack confers immunity and when inoculation is not fatal immunity results.

Flexner and Noguchi isolated a minute coccus and cultivated it anaerobically in fluid serum. They believe this organism the specific cause of the disease but their view has not been generally accepted.

Dixon and Rucker also succeeded in isolating a similar but probably not identical organism from the brain and cord of patients dead of the disease. At first they were inclined to believe it the causative organism but later rejected it as the specific etiological factor. Recently Rosenow and others have obtained cultures of an organism described as **pleomorphic streptococcus** from the nasal mucosa, the pharynx and tonsils of poliomyelitis patients. They have isolated the same organism from the ventricular fluid, brain and cord of fatal cases.

The **pleomorphic streptococcus** is described as a coccus arranged in pairs like pneumococci (but without a capsule) and in chains. Marked variations in size are observed. In dextrose-ascitic broth growth occurs under aerobic and anaerobic conditions but is more rapid in an aerobic atmosphere.

Incubated at body temperature growth appears in from 3 to 5 days and continues for several weeks. Old cultures show large and club-shaped forms. Young cultures are Gram positive and old cultures Gram negative.

Growth occurs on blood-agar incubated at body temperature, aerobically. Some describe the colonies on this medium as small, white, and dry, without any change in the appearance of the underlying or surrounding medium; others describe a slight clear zone or greenish zone surrounding each colony.

Various observers have reported inoculation of rabbits by placing pleomorphic streptococcus upon the nasal mucosa, by injecting it subcutaneously, by injecting it into a nerve or beneath the dura. In such cases symptoms and pathological changes associated with the pleomorphic streptococcus and recovery of the organism have convinced Rosenow and others that it is the specific cause of poliomyelitis but at present we must consider their case not proven.

Netter has discovered that the blood serum of persons recovered from poliomyelitis possesses distinct therapeutic value, practically undiminished throughout their life. He recommends the intraspinal injection of such serum in the treatment of the disease, from 5 to 13 cc. each day for 1 week.

SPIROCHÆTA ICTEROHEMORRHAGA

(Spirochæta Nodosa)

The discoveries recently reported by Ito, Matsuzaki, Uhlenhuth and Fromme seem to indicate that Weil's disease is caused by infection and that the offending organism is a small spirochete.

Ito and Matsuzaki have described three closely allied strains of spirochæta icterohemorrhaga that apparently are the same as spirochæta nodosa.

This organism (or organisms) has been found constantly associated with Weil's disease. Blood cultures, especially when made on the fourth or fifth day of the disease are usually positive. The organism is occasionally present in the sputum, urine, or feces of patients during the disease and sometimes as long as 50 days after recovery.

Inoculation of mice, rats and guinea-pigs with cultures obtained from patients causes disease the same as in man and identical to the disturbance produced by inoculation of these animals with the urine of patients.

Virulent spirochæta are also present in the urine and feces of inoculated animals.

Spirochæta icterohemorrhaga is anaerobic and facultative aerobic. Uhlenhuth and Fromme cultivate it in tubes of rabbit serum diluted with normal salt solution and cover this medium with a layer of oil to exclude air.

The serum of persons and animals, after recovery from Weil's disease, contains antibodies which will produce Pfeiffer's phenomenon when brought into contact with spirochæta icterohemorrhaga.

GANGRENE

The type of gangrene due to infection has been attributed to several bacilli that were looked upon as most constantly associated with the disease and, therefore, probably a specific factor.

It is now well established that gangrene due to infection may be produced by a variety of organisms, the majority of which are obligate anaerobic.

Small pox, scarlet fever, yellow fever and measles are all infectious diseases caused by unknown microörganisms.

Small Pox.—The virus of this disease is present in the pustules that develop on the skin of infected persons. One attack of the disease confers immunity. Cow pox is a disease of cattle similar to small pox in man, and the virus of cow pox is present in the pustules which develop on the epithelial surface of infected animals. In 1798 Jenner discovered that the virus taken from the pustules of cow pox and applied to the skin of human beings would inoculate them producing a mild infection which immunized them against small pox.

Scarlet Fever.—Although streptococci are constantly associated with this disease, the majority of the medical profession does not believe that they are the specific etiological factor. The virus of scarlet fever is present in the blood in the early days of the disease and is believed by many to also be present in the desquamated epithelium.

Yellow Fever.—The virus of this disease is present in the blood of affected persons as has been proven by withdrawing such blood and injecting it into healthy persons, thereby inoculating them. This virus is never transferred directly from man to man. Man is always infected through the bite of a mosquito. The mosquito in turn acquires the organism by feeding on the blood of yellow fever patients.

Measles.—As shown by Anderson the virus measles is present in the nasal secretions and the circulating blood prior to the development of the rash.

Anderson's work has clearly shown that this virus is transmitted from man to man by direct intimate contact during the period of coryza and before the rash develops.

There is much evidence to indicate that scarlet fever is probably transmitted in a similar manner.

BACTERIOLOGY

PART II

CHAPTER I

EXAMINATION OF WATER

Water which may be considered potable, without suspicion, contains few or no bacteria and no pathogenic bacteria (no colon bacilli). Most water contains some bacteria. That which shows 100 colonies per cubic centimeter or less, none of which are pathogenic, should be considered safe. When 500 colonies per cubic centimeter or more are present, even though none of them are pathogenic, it must be considered with suspicion, because where so much bacterial life can be found in water, as a rule, conditions are favorable for the entrance of disease-producing germs at any time.

Colon bacilli in water are not always of human origin; in populated districts they usually are and hence we are compelled to consider the presence of colon bacilli in water evidence of dangerous contamination.

Colon bacilli in water, of themselves, probably do not constitute a grave danger, but experience has shown that, as a rule, colon bacilli in water indicates pollution with human sewage, and such pollution eventually results in an epidemic of typhoid fever, cholera or other water-borne diseases.

It is always difficult, frequently impossible, to detect and isolate typhoid bacilli and other pathogenic bacteria from water, even when such organisms are known to be in it. The colon bacillus can be detected and isolated with comparative ease; hence, water is examined for colon bacilli and judged as good or bad, according to the presence or absence of the colon bacillus.

Efficient chemical treatment or filtration not only removes or destroys all pathogenic bacteria and colon bacilli, it removes or destroys more than 95 per cent. of the total bacterial content. Hence, in estimating the efficiency of chemical treatment or filtration, samples are obtained before and after treatment and examined to determine the number of bacteria per cubic centimeter, also for colon bacilli.

To get a fair sample for examination, when water is obtained from a tap or faucet, the water should be allowed to flow for at least 5 minutes before collecting a specimen.

If it is to be obtained from a spring, lake or stream the person collecting should wash his hands and then submerge the container before opening it. The container should be kept submerged until it is full and has been closed or stoppered.

When a sample has been obtained it should be examined as soon as possible. If time must elapse before examination, the sample should be cooled to 15°C. and maintained at or below that temperature until examined.

Samples for bacteriological examination are collected in sterile receptacles, measured with sterile pipettes and cultured in sterilized tubes and dishes.

TECHNIQUE

Fermentation Test.—Shake container to evenly distribute bacteria that may be in the water. Take a number of fermentation tubes containing lactose broth medium.

Put 0.1 cc. into each of the first two tubes.
Put 0.2 cc. into each of the second two tubes.
Put 0.4 cc. into each of the third two tubes.
Put 0.8 cc. into each of the fourth two tubes.
Put 1.5 cc. into each of the fifth two tubes.
Put 3.0 cc. into each of the sixth two tubes.
Put 6.0 cc. into each of the seventh two tubes.
Put 10.0 cc. into each of the eighth two tubes

Place one set of tubes containing the various amounts of water in an incubator at 37°C. and the others in an incubator at room temperature.

Examine these tubes at 24-hour intervals for 3 days.

If fermentation occurs gas accumulates at the top of the tube. When fermentation occurs it is due to the presence of the colon bacillus in the majority of cases, but one must remember that other organisms can, and occasionally do, cause fermentation even when the water is free of colon bacilli, usually bacillus Welchii.

Plating.—A number of tubes of litmus lactose agar are liquefied by placing in a water bath and heating to the boiling point. They are then cooled to 45°C.

Put 0.1 cc. of water into the first tube and empty tube into Petri dish.
Put 0.2 cc. of water into the second tube and empty tube into Petri dish.
Put 0.4 cc. of water into the third tube and empty tube into Petri dish.
Put 0.8 cc. of water into the fourth tube and empty tube into Petri dish.
Put 1.5 cc. of water into the fifth tube and empty tube into Petri dish.
Put 2.0 cc. of water into the sixth tube and empty tube into Petri dish.

Make a duplicate set of plates, using 2 or 3 per cent. plain agar having a reaction of +1.5. Incubate one set at room temperature, the other at 37°C.

Count the colonies every 24 hours for 3 days.

When water is dropped into a tube of agar the tube should be inverted several times to evenly distribute the water before pouring into a Petri dish. Care should be taken to distribute the liquid agar evenly over the entire bottom of the Petri dish and the dish should not thereafter be tilted, moved nor placed in incubator until the agar has solidified.

The colon bacillus and other organisms that ferment lactose turn litmus lactose agar red. When red colonies appear they are removed from the plates with a sterile platinum loop and planted in Dunham's solution, litmus milk, and on plain agar and gelatin. The Dunham's solution is tested for indol and the morphology, motility and reaction with Gram's staining observed. If necessary further differential cultures may be made.

The above technique should be employed when examining a new or unknown sample of water. Samples of water obtained from the same source at frequent intervals, as in routine work, number of examinations showing that the bacteria contained does not vary to any great extent, do not need to be planted in so many different quantities. With water which is usually potable, it is sufficient to plant just three different quantities in fermentation tubes—3 cc., 5 cc. and 10 cc.—and three different quantities in plates—1 cc., 1½ cc., and 2 cc.

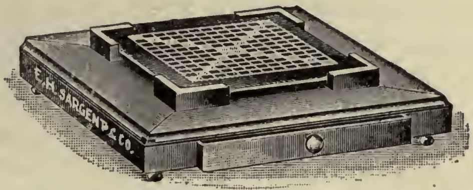
E. H. SARGENT & CO.

Fig. 35.—Wolfhügel's Colony Counter.

Water which is grossly contaminated with sewage is frequently as rich in bacteria as dirty milk and hence must be diluted with sterile distilled water before plating or making fermentation tubes.

For reasons discussed on page 181 it is essential in making a numerical count to obtain plates that show between 20 and 200 colonies. When making the count all colonies that are visible when examined through a reading lens that magnifies three diameters are to be counted.

When water is suspected to contain the cholera spirillum, to 100 cc. of the suspected water add 1 Gm. peptone (Witte's) and ½ Gm. sodium chloride, shake to mix and incubate at 37°C. in a tall, narrow container. After 6 hours carefully remove several loopsful from the top and transplant as when examining feces (pages 116 and 117).

CHAPTER II

EXAMINATION OF MILK

Milk is exceptionally liable to contamination with bacteria and is a most favorable medium for their development. Consequently practically all of it has an abundant bacterial content.

Milk that shows 20,000 colonies per cubic centimeter or less is extraordinarily clean. Milk that shows 10,000 colonies per cubic centimeter or less is seldom observed.

In winter, average city milk shows from 5000 to 500,000 colonies per cubic centimeter, in summer twice as many or more. Milk that shows 1,000,000 colonies per cubic centimeter or more is dirty and has been carelessly handled.

Before examining, milk must be thoroughly shaken to evenly distribute the bacteria.

Fermentation tests are not made as when examining water, because fermenting organisms are known to be always present, most of them are lactic acid bacilli (non-pathogenic) and some are colon bacilli from the cow.

Various disease-producing bacteria may be contained in milk, including staphylococci, streptococci, diphtheria bacilli, tubercle bacilli and typhoid bacilli; the tubercle bacillus is the most frequently present pathogenic organism and the streptococcus probably comes next. From 6 to 13 per cent. of all market milk contains tubercle bacilli.

Many observers believe that most of the epidemics of streptococcus throat infections are derived from milk.

It is practically impossible to isolate tubercle bacilli or diphtheria bacilli from milk, or to observe them in it. For the detection of these organisms in milk inoculations must be made, and these sometimes fail to disclose them. Animal inoculations are generally omitted in the routine examination, opinion as to the bacterial content being based upon examination of sediment for leucocytes and bacteria, and plating to determine the number of bacteria per cubic centimeter.

For the study of leucocytes in milk and observing the presence of staphylococci and streptococci, the best and quickest is Stewart's method.

When observing the leucocytes one should know whether the milk has been heated or pasteurized because heating apparently increases the number.

TECHNIQUE

Shake the sample to evenly distribute contained bacteria.

Take 1 cc. of the milk and mix with 9 cc. of sterile distilled water, dilution No. 1 equals 1 : 10; take 1 cc. of dilution No. 1 and mix with 99 cc. of water, dilution No. 2 equals 1 : 1000; take 1 cc. of dilution No. 2 and mix with 9 cc. of water, dilution No. 3 equals 1 : 10,000.

Take 1 cc. of dilution No. 2 and mix with 99 cc. of water, dilution No. 4 equals 1 : 100,000.

Liquefy a number of tubes of plain 2 to 3 per cent. agar (+ 1.5 reaction) and cool to 45°C.

In each of the first two tubes put 1 cc. of 1 : 1000 dilution and plate.

In each of the second two tubes put 0.5 cc. of 1 : 1000 dilution and plate.

In each of the third two tubes put 2 cc. of 1 : 10,000 dilution and plate.

In each of the fourth two tubes put 1 cc. of 1 : 10,000 dilution and plate.

In each of the fifth two tubes put 0.5 cc. of 1 : 100,000 dilution and plate.

Incubate one set at room temperature, the other at 37°C. for 3 days and count colonies every 24 hours.

Leucocytes.—Fill a Stewart's tube (1 cc.) with milk, insert rubber plug in one end and push the rubber bulb down tight on the other. Place in centrifuge with plugged end at periphery. Spin at 3000 revolutions per minute for 5 minutes. Hold the tube horizontal and remove plug without scraping against edge of tube. Smear the sediment collected on the plug over an area of 1 square centimeter on a glass slide by rubbing the plug against it.

Fix by heating over flame and stain with Loeffler's methylene blue for 5 minutes. Dry and examine under oil immersion lens.

The leucocytes are counted in 10 fields. If a total of 230 or more leucocytes is observed in unheated milk it is condemned; when less than this number are observed associated with considerable numbers of streptococci it is condemned.

When making this examination many fields will be observed in which there are no bacteria, fields that contain them will only show a few—if the milk is clean—less than 500,000 colonies per cubic centimeters when plated. If most of the fields show a few bacteria plating will show more than 500,000 colonies per cubic centimeter, and if numerous fields show large numbers or clumps of bacteria, the milk is very dirty and will show more than 1,000,000 colonies per cubic centimeter.

ANIMAL INOCULATION TESTS

Place 50 cc. of milk in centrifuge tube and spin at high speed, 3000 revolutions per minute or more, for 10 minutes. Remove the cream, place it in a centrifuge tube. Remove the skimmed milk without disturbing the sediment in the bottom of the tube. Fill both the centrifuge tubes up to the 50-cc. mark with sterile distilled water, shake thoroughly to evenly mix the contents of each tube, then centrifugalize them as before. Remove the upper 48 cc. of fluid from each tube without disturbing the sediment. Suck the remaining 2 cc. of fluid and sediment from the cream into a syringe, and inject into the peritoneal cavity of a guinea-pig. In like manner inoculate a second pig with the sediment from the skimmed milk.

Healthy pigs, weighing between 200 and 300 Gm. should be used.

Wholesome milk will not injure guinea-pigs. If the animals remain alive, as they should if the milk does not contain large numbers of pathogenic organisms other than tubercle bacilli, kill them 6 weeks after inoculation and examine for tuberculosis. It requires a month or more for tubercle bacilli to produce distinct, characteristic lesions in guinea-pigs.

CHAPTER III

EXAMINATION OF FLUIDS AND SOLIDS

Fluids other than milk are subjected to bacteriological examination for several purposes: (1) to determine whether supposedly sterile fluids are so, and if not the number, perhaps also the kind, of bacteria contained: (2) to determine the character of bacteria present in fluids known to contain bacteria.

TECHNIQUE

Shake the sample thoroughly, take a large drop and spread it on a slide and fix by gently heating until dry, stain with methylene blue and examine microscopically. If no bacteria are observed or if only a few are seen in some fields and none in others, plating will probably show less than 500,000 colonies per cubic centimeter; if many bacteria are observed in most of the fields, plating will show millions per cubic centimeter. Such a preliminary inspection, together with other known facts, usually give one an idea as to the proper amount of dilution required for plating.

In order to make accurate counts, not more than 200 colonies should develop on a plate, and to obtain this, the richer a substance is in bacteria the more it must be diluted, the smaller the amount placed in the plate.

To remove individual colonies from a plate containing various organisms, in order to further study the organisms separately and to identify them, the colonies must be discrete and sufficiently isolated from each other to permit the removal of one colony with a loop, without touching any other colony.

Having estimated the degree of dilution required, the fluid is diluted and plated the same as when examining water or milk.

Plates should be made on three different media, plain agar, litmus lactose agar and gelatin. The gelatin plates are incubated at room temperature, and the rest at 37°C.

Substances, both fluids and solids, contaminated with bacteria may contain the tetanus bacillus. As this organism is an obligate anaerobe, several plates should be incubated under anaerobic conditions.

If in addition to a determination of the number of bacteria present, the species must be determined, a representative plate is selected after making the count, and one of each different kind of colonies observed is removed and plated separately. If pure cultures develop on the second series of plates, further subcultures are made on various media sufficient to establish the identity of the organisms. If the second series of plates do not show pure cultures, colonies are removed from them and again plated until a pure culture is obtained.

Solids are examined for the same reasons and usually in the same way as fluids, except that they are first dissolved or suspended in sterile distilled water.

If the material under examination is suspected of being nocuous for guinea-pigs, 2 cc. of it should be injected into the peritoneal cavity of a guinea-pig.

EXAMINATION OF QUININE

Quinine salts which are intended for hypodermic administration should be examined for bacteria, especially the tetanus bacillus, as cases of tetanus following the hypodermic administration of quinine have been reported.

TECHNIQUE

Place 1 Gm. of quinine in each of two flasks containing 1000 cc. of glucose bouillon. Incubate at 37°C. one flask in an aerobic atmosphere, the other in an anaerobic atmosphere. Inspect daily, and if growth occurs centrifugalize 15 cc. of the culture at high speed for 20 minutes, collect the bottom cubic centimeter, and inject it subcutaneously into a guinea-pig.

Subcutaneous injections of sublethal doses of an aqueous solution of the quinine should also be made.

EXAMINATION OF CAT-GUT

Cat-gut is especially apt to contain tetanus spores before treatment and occasionally does after attempts at sterilization by approved methods, hence cat-gut must always be proved sterile before it is dispensed for use in surgery.

TECHNIQUE

Select several strands or packets from a lot and put in a large test-tube containing about 50 cc. of glucose bouillon, incubate at 37°C. in an anaerobic atmosphere. Inspect daily, and if growth does not appear in 5 days it is sterile. Some tubes should also be incubated in an aerobic atmosphere, and if desired guinea-pig inoculations may be made.

EXAMINATION OF KETCHUP

ESTIMATION OF MOLDS

A drop of the product to be examined is placed on a microscope slide and a cover glass is placed over it and pressed down till a film of the product about 0.1 millimeter thick is obtained. After some experience this can be done fairly well. A film much thicker than this is too dense to be examined successfully, while a much thinner film, necessitates pressing the liquids out, which gives a very uneven appearing preparation. When a satisfactory mount has been obtained, it is placed under the microscope and examined. The power used is about 90 diameters, and such that the area of substance actually examined in each field of view is approximately 1.5 square millimeters.

A field is examined for the presence or absence of mold filaments, the result noted, and the slide moved so as to bring an entirely new field into view. This is repeated till approximately 50 fields have been examined, and the percentage of fields showing molds present are then calculated. Our experience has demonstrated that for homemade ketchups this is practically zero, and with some manufactured ketchup it is as low as from 2 to 5 per cent., while for carelessly made products it may be 100 per cent.; that is, every field would show the presence of mold. Investigations under factory conditions clearly indicate that with only reasonable care the proportion of fields having molds can be kept below 25 per cent. A specimen in which 60 per cent. of the fields have molds is in more than twice as bad a condition as one containing 30 per cent.

After the percentage reaches 30 to 40 per cent. it will be found that some of the fields frequently have more than one filament or clump of mold, and the number of such fragments might be counted, but in this laboratory this usually is not done. A Thoma-Zeiss counting cell with a center disk of 0.75 inch instead of 0.25 inch, as usually furnished, would give a regular depth of liquid and would be more exact than the method described, but this must be specially manufactured, not being listed in any of the catalogues of microscopic supplies, and the method as given is sufficiently accurate for the purpose. When the number of fragments of mold per cubic centimeter is estimated, it has been found to range from virtually zero to over 20,000. There is no excuse for a manufacturer allowing such conditions to prevail that his ketchup shows more than 2000 per cubic centimeter, while some manufacturers by careful handling hold it down to 150.

ESTIMATION OF YEASTS AND SPORES

Though the spores referred to are those coming from molds and correspond to seeds in more highly developed plants, it is frequently very difficult to differentiate some of them with certainty from some yeasts without making cultures, which is obviously impossible in a product that has been sterilized by heat. For this reason the yeasts and spores have been reported together, and if there seemed to be a larger percentage of the latter, mentioned was made of that fact.

To make a count 10 cc. of the product is thoroughly mixed with 20 cc. of water, and after being allowed to rest for a moment to permit the very coarsest particles to settle out, a small drop is place on the central disk of the Thoma-Zeiss counting cell and then covered with a glass. Care must be exercised to have the slide perfectly clean, so that, when the cover glass is put in place, a series of Newton's rings* results from the perfect contact of the glass surfaces; and, furthermore, the drop should be of such size as not to overrun the moat around the central disk and creep in underneath the cover glass, thus interfering with the contact.

With the magnification of 180, it has been the practice in this laboratory to count the number of yeasts and spores on one-half of the ruled squares on the disk. With the dilution used this calculates back to a volume equal to $\frac{1}{60}$ cubic millimeter in the original sample, and reports are made on that basis rather than on the number in a cubic centimeter, because the former number is moreadily grasped by the mind and affords a simpler notation. To obtain the numbers per cubic centimeter the count made is simply multiplied by 60,000.

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* These are rainbow-colored rings produced at the point of contact when polished plates of glass are pressed against each other.

It has been found in practice that the number of yeasts and spores varies, for 1/60 cubic millimeter, from practically none in home-made and first-class commercial ketchups up to 100 or 200, and in one sample the number was as high as 1200. Laboratory experiments show that, when the number of yeasts in raw pulp reaches from 30 to 35 in 1/60 cubic millimeter the spoilage may frequently be detectable by an expert by odor or taste, and from experiments made under proper factory conditions, it seems perfectly feasible to keep the number in commercial ketchups below 25.

ESTIMATION OF BACTERIA

The bacteria are estimated from the same mounted sample as that used for the yeasts and spores. A power of about 500, obtained by using a high-power ocular, is employed in this case, and because of the greater number present a smaller area is counted over. Usually the number in several areas, each consisting of five of the small-sized squares, is counted and the number of organisms per cubic centimeter is calculated by multiplying the average number in these areas by 2,400,000. Thus far it has proved impracticable to count the micrococci present, as they are likely to be confused with other bodies frequently present in such products, such as particles of clay, etc. A comparison of this method with the ordinary cultural methods on samples in which the organisms had not been killed has almost invariably shown that the one used gives too low instead of too high results. In some cases it was found to give not more than one-third of the entire number present. The estimates of the laboratory on this point may, therefore, be considered very conservative.

As regards the limits which may be expected in the examination of ketchups for bacteria, it might be stated that some manufactured samples as well as good, clean products made by household methods, have been examined, and the count found to be so low when estimated by this method that the numbers present were reported as negligible. In other words, it was found that for the areas counted over, the number of bacteria averaged less than one—that is, less than 2,400,000 per cubic centimeter. It is unusual, however, for the final number per cubic centimeter to be less than from 2,000,000 to 10,000,000 organisms. Contrasted with this number as a minimum, it has been found that the number has occasionally exceeded 300,000,000 per cubic centimeter. Such a number as this would indicate extremely bad conditions and carelessness in handling, as the studies of factory conditions has shown that there is little excuse for the number ever exceeding 25,000,000 per cubic centimeter. While experiments have also shown that although the effect produced by the bacteria on the product varies with different species, it is true that their presence can frequently be detected in the raw pulp by odor or taste when the number exceeds 25,000,000 per cubic centimeter and sometimes when the count is as low as 10,000,000.

To one who has not been initiated into the mysteries of the microscope the presence of such a number of bacteria in a food product seems inexcusable. It must be remembered in this connection that most of these are probably nonpathogenic forms, and that many occur naturally on the skins of the fruits. It does not seem just to set a standard so high as to virtually prohibit the manufacture of the product under commercial conditions; rather, the idea is to set a limit that the manufacturer can attain if due care is exercised, and which will insure a cleanly product. It is, however, perfectly possible to make a cleanly, wholesome product commercially, even though the number of bacteria exceed that in the home-made article.

The allowable limits for the bacterial content of tomato pulp vary according to the concentration. The number, however, should be low enough so that when the amount of concentrating necessary for its conversion into ketchup has been accomplished the final product will still be within permissible limits (25,000,000 per cubic centimeter). Thus for a pulp which must be concentrated one-half the bacterial counts should not exceed about half the limits stated above for the ketchup itself—*i.e.*, it should not be more than 12,500,000 per cubic centimeter. The same general rule should also apply to the content of molds and of yeasts.

To insure a sound product, free from decay or any filthy material, many factors must be carefully watched, for not infrequently oversight in one particular has been found to have undone the good effects of the care exercised in all other ways. Thus it is possible for the washing of the fruit to be ideal and the sorting out or removing of the decayed portions beyond criticism, and yet a delay in making up the pulp into the final product may allow an amount of decomposition to occur which offsets the care previously exercised. It has been a matter of surprise to some manufacturers to find with what rapidity some of these organisms increase. In one factory where this point was tested, the bacterial content in a batch of tomato trimming juice was found to be about 7,000,000 per cubic centimeter when taken from the peeling tables, and after standing at room temperature for 5 hours it had increased to 84,000,000. This was a twelvefold increase in a length of time which was less than half the working day for some of the factories visited. At the end of 5 days the number had increased to nearly 3,000,000,000 per cubic centimeter. Thus it is seen that delay in manufacture is very liable to result disastrously.

Such facts as these serve to emphasize the great importance of absolute cleanliness in every detail about factories of this kind. Dirty floors and ceilings and apparatus left with residues of tomato product clinging to them are most fruitful sources for the contamination of new batches of the product. To clean such an establishment properly it is almost imperative that machinery and woodwork be washed by means of live steam used lavishly at frequent intervals. To leave buckets, tables, conveyors, or any other part of the equipment or floors overnight without cleansing them, as was the practice in some factories, is reprehensible and tends to contaminate the product and lead to spoilage and loss.*

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* From Bulletin, Feb. 12, 1911, U. S. Department of Agriculture, "Tomato Ketchup Under the Microscope," by B. J. Howard, Chief, Microchemical Laboratory.

EXAMINATION OF EGGS

When laid, eggs contain a few bacteria; if the shells have no cracks or breaks in them and are free of macroscopic particles of dirt and if such eggs are collected within several hours after they have been laid and immediately stored in clean, dry containers at about 15°C., the eggs remain "fresh" for many weeks with slight, if any, multiplication of contained bacteria.

Bacteria rapidly increase in cracked eggs and those exposed to a temperature of 30°C. to 40°C.

Market eggs, shown by candling, chemical examination and diet tests, to be palatable and nutritious, yield 20,000 to 200,000 colonies per gram when cultured a small portion of which are colon bacilli.

Eggs in the shell that show more than 1000 colonies per gram are not acceptable as food.

Frozen eggs and desiccated eggs (either whites, yolks or whole eggs) always show a relatively more abundant flora than eggs in the shell.

Frozen and desiccated eggs may be considered acceptable when the cultural test shows less than 2,000,000 colonies per gram, not more than 0.02 per cent. of which are colon bacilli. When the percentage of colon bacilli is considerably greater or when the total count is above 20,000,000 colonies per cubic centimeter, the eggs have been handled, canned or stored in a manner that makes them dangerous foodstuff and when the count is above 20,000,000 in addition to being dangerous to health, they have been robbed of a considerable portion of their nutritive value.

These figures and conclusions are based on a résumé of the extensive original investigations of R . C. Rosenberger.

TECHNIQUE FOR EXAMINATION OF EGGS IN SHELL

1. Wash the eggs in 5 per cent. phenol solution at 50°C. by gently sponging with cotton.

2. Rinse in sterile water.

3. Break shell with sterile spatula and empty contents into wide-mouthed, sterile, weighed Erlenmeyer flask.

4. Determine weight of egg or eggs in flask and add an equal volume of sterile water, shake until homogenized.

5. Put 1.0 Gm. in flask and mix with 499 Gm. of sterile water (1 : 1000 dilution).

6. Mix 1 cc. of the 1 : 1000 dilution with 9 cc. of sterile water and plates of plain agar and litmus lactose agar with the following quantities: 0.1 cc., 0.2 cc., 0.5 cc., 0.7 cc. and 1.0 cc.; also plant 0.1 cc., 0.5 cc. and 1.0 cc. in fermentation tubes containing litmus lactose broth.

Incubate at 37°C. for 3 days and inspect each day. Subculture representative red colonies that appear on litmus agar and make differential tests for identification of colon bacillus.

FROZEN EGGS AND DESICCATED EGGS

1. Weigh out 1.0 Gm. of egg on a sterile watch crystal, put in sterile flask with 99 cc. of sterile water, shake until entirely dissolved and thoroughly mixed.

2. Transfer 1 cc. of first dilution (1 : 100) to a second flask containing 99 cc. of sterile water and thoroughly mix, making a 1 : 10,000 dilution.

3. Plant both plain agar and litmus lactose agar plates with the following quantities of the second dilution: 0.1 cc., 0.2 cc., 0.5 cc., 0.7 cc. and 1.0 cc.

4. Incubate, inspect and count as when examining eggs taken from shell.

CHAPTER IV

DETERMINATION OF THE GERMICIDAL POWER OF CHEMICAL DISINFECTANTS

To accurately determine the amount of a particular chemical required to kill all pathogenic bacteria, under varying conditions of environment, is practically impossible. The germicidal power of a chemical disinfectant varies in different media and at different temperatures; some which are powerful germicides in media free of albumin are nearly inert when associated with albumin; some are powerful germicides when in solution and less germicidal when mixed with a substance that throws them out of solution.

An estimation of the germicidal power of a given substance may be made which gives valuable information regarding the proper employment of that substance as a disinfectant under various conditions.

This is done by obtaining typical pure cultures of as many different organisms as possible—staphylococci, typhoid, colon, diphtheria, tubercle, anthrax and tetanus bacilli, etc.—mixing different amounts of the disinfectant with a fixed amount of the bacteria, both with and without the admixture of organic matter, letting the mixture stand at a given temperature for a certain time and then testing the mixture for sterility by making subcultures from it.

This is a very extensive, time-consuming procedure and at best yields only approximate results. Different strains of the same organism under identical conditions often show variations of several hundred per cent. in their resistance to a chemical disinfectant.

Within certain limits the following conditions influence the action of chemical disinfectants:

1. Length of time the disinfectant is in contact with bacteria.

2. Temperature at which the contact occurs (at low temperatures disinfectants are much less active than at high, for every rise of 10° the efficiency is increased two- to tenfold.

3. Physical and chemical properties of the medium in which the infective organisms exist.

4. The amount of disinfectant and the number and nature of the bacteria.

On account of the many factors that complicate and limit approximate determinations of the disinfectant power of chemicals, such determinations were largely neglected until recent years with a consequent development of gross misconceptions and inefficient use of chemical disinfectants.

In 1898 Rideal and Walker introduced a test, known as the "Rideal-Walker Carbolic Acid Coefficient Test," for the determination of the relative disinfectant power of chemicals, in comparison with that of carbolic acid. Later this test was improved upon by the introduction of one known as the "Lancet

Method," and still later Anderson and McClintic introduced the "Hygienic Laboratory Method."

None of these methods is ideal; they are difficult and time-consuming and yield only approximate results, yet they are of great value as approximate and relative knowledge of disinfectant value is by far superior to total ignorance.

Of the various methods, the "Hygienic Laboratory Method" is unquestionably the best; especially in the United States it should always be the method of choice. In "Hygienic Laboratory Bulletin, No. 82, of the Public Health and Marine Hospital Service of the United States," April, 1912, Anderson and McClintic describe their test in a clear, concise manner. Those intending to make coefficient tests should obtain the above-mentioned bulletin. The following description of the tests is quoted from Anderson and McClintic:

PRINCIPAL FACTORS INVOLVED IN THE EXAMINATION OF DISINFECTANTS

"Lack of attention to the different factors concerned in the examination of disinfectants is responsible for a large percentage of the inconsistencies or discrepancies in results obtained by the same or by different workers when working with the same disinfectants. Unless strict attention is paid to the various influences involved it is useless to expect to find any method satisfactory.

In order to better emphasize the effect of these influences upon the results obtained, the various factors involved will be discussed under the appropriate headings.

TEST ORGANISM

Unless different observers use the same species of organism there can be no possibility of uniformity in results. The coefficient obtained with different species may vary as much as 300 per cent. For this reason it is important that one species be selected for use as the test organism. It would be highly desirable if the same strain of this species could be used by all workers in the testing of disinfectants, as there is often a variation in the resistance of different strains of the same species. This objection does not apply as much to the typhoid organism as to the colon bacillus, and to some other bacteria.

We made a number of comparative tests with different strains of B. typhosus and B. coli and found a very much greater difference in the resistance of different strains of the colon bacillus than of the typhoid bacillus.

It is most important that, before being used for a test, the organism be carried over on broth daily for at least 1 week. In all cases a 24-hour culture should be used, as there is decided difference in the resistance of a 24-hour and a 48-hour culture, the latter being the more resistant.

In order to avoid clumps in the culture the 24-hour broth culture should be well shaken and then filtered through sterile filter-paper into a sterile test-tube. After this it should be placed in the water bath in order that it may reach the standard temperature before being added to the disinfectant dilutions.

TEMPERATURE OF EXPERIMENT

It is a well-known principle in the use of disinfectants that, within certain limits, the higher the temperature at which the disinfectant is used the greater are its germicidal properties. This increase in the germicidal properties of disinfectants through the influence of heat is not the same for all disinfectants; some, such as formaldehyde, are more strongly influenced than others. It will be seen that at 15°C. phenol, in dilution of 1:80, killed the typhoid organism in 2½ minutes, while at a temperature of 30°C. the organism was killed in the same length of time by a dilution of 1:120.

On account of the great variation of temperature in the United States, especially during the summer, it becomes necessary that a standard temperature be adopted. We have adopted a temperature of 20°C. and have devised a simple water bath to be used for maintaining this temperature. This bath consists of a wooden box 20 inches deep, 21 inches long, and 21 inches wide. Inside this box a 14-quart agateware pail 10 inches deep is placed and sawdust is well-packed around, sufficient being placed on the bottom of the box to bring the rim of the pail on a level with the top of the box.

A tightly fitting wooden cover is placed over the pail, so made that the edges project slightly over the rim. In the cover are a sufficient number of holes for the seeding tubes, a thermometer, and the tube containing the culture. About 3 inches below the rim of the pail a false bottom of wire gauze is placed; this is for the seeding tubes, etc., to rest on. Water is placed in the pail to within ½ inch of the top.

When an experiment is to be made the temperature of the water in the pail is taken, and if above or below 20°C. it is brought to the desired temperature by the addition of either cold or hot water. It will be found that only very slight change takes place in the temperature of the bath in an hour and that it is an easy matter to keep the temperature of the bath at the figures desired. It is of advantage, in regulating the temperature of the bath, to have a spigot in the bottom of the pail to draw off the water when so desired.

PROPORTION OF CULTURE TO DISINFECTANT

As disinfection is the result of chemical action of the disinfecting agent upon the test organism, mass action is an important factor in the testing of disinfectants. By this is meant that within certain limits the greater the number of bacteria added to the disinfectant dilution the stronger the dilution required to do the same work. For this reason it is important that the amount of culture to be added to the dilution should be stated in definite quantities and not in "drops" or in "spoonfuls." We have adopted the practice of using 0.1 cc. of a 24-hour broth culture. For measuring this we use a delivery pipette graduated in tenths.

MEDIA FOR SUBCULTURES

There is probably no one factor, with the possible exception of temperature, that has more to do with irregularities in results than the media for subcultures. Where the typhoid bacillus is used for the test organism, as in the Rideal-Walker method, and the method proposed by us, it is of paramount importance that the media have a reaction of just +1.5. A reaction greater than this exerts a decided inhibiting action upon the growth of the transplanted organism. This is an important point, for if the transplant is made from a test dilution which is just under the killing strength of the disinfectant, the inhibiting action of the media may be sufficient to prevent growth, thus giving a false result. In the hands of different workers a difference in the reaction of the media may result from the degree to which the color reaction in titration is carried. We always carry it to the point where the pink color is distinctly perceptible, but even then there seems to be at times a slight difference in various lots of our media.

It is a noteworthy fact that the influence of the reaction of the subculture media upon the growth of the exposed organism was decidedly more pronounced after it had been exposed to phenol than to any of the other disinfectants tried.

It was found, too, that a more vigorous growth and a growth from stronger solutions were obtained when the exposed organisms were planted in meat broth than when they were planted in extract broth. It is therefore evident from the above that the reaction and character of the subculture media has an important bearing upon the results obtained in determining the phenol coefficient of disinfectants. However, as extract broth is more uniform in composition, more easily prepared, and cheaper than meat broth, we recommend that extract broth be always used and when it is not, that the fact be so stated.

The amount of media in the tubes for subculture should be sufficient to prevent any antiseptic action, due to the transferred disinfectant. With some substances, such as bichloride of mercury, this is often an important point.

It may be stated here that in our work with some disinfectants, particularly those containing coal-tar products, the disinfectant carried over in making the inoculations of the subcultures caused a distinct cloudiness in the media; but after 48 hours' incubation this always cleared up so that there was no difficulty in making out the presence or absence of growth.

MacConkey's bile-salt medium was given a limited trial with B. coli communis. We found that after exposing the B. coli communis to the action of a 1 per cent. solution of carbolic acid and planting in MacConkey's medium and extract broth, respectively, every 2½ minutes for 15 minutes, and incubating for 48 hours, there was a growth in all the tubes of the extract broth, but only in a 2½-minute tube of MacConkey's medium. This condition or result was more marked with carbolic acid than with any other disinfectant tried.

When using the B. typhosus the possibility of contamination in the tubes of broth that show a growth at the end of 48 hours can be determined by the use of antityphoid serum.

ORGANIC MATTER

Under practical conditions disinfectants are commonly used in the presence of more or less organic matter. It is therefore very important to know to what extent the germicidal efficiency of a disinfectant is affected by the presence of organic matters.

The character and quality of organic matter present as well as its effect upon the germicidal efficiency of different disinfectants are such widely varying factors that the standardization of disinfectants in the presence of organic matter is a rather difficult problem. We have done a great deal of experimental work with disinfectants in which various kinds of organic matter were tried, and it has been difficult to find a form of organic matter that is entirely satisfactory for standardization purposes. Urine, blood serum, dead (killed by heat) broth cultures of the typhoid bacillus, peptone, gelatin, egg albumen, starch, etc., were all given a trial and we have finally decided to use a mixture of an aqueous solution of peptone and gelatin.

It is manifestly necessary that any form of organic matter, used for standardization of disinfectants, be of known, definite composition. Unless this is the case only widely varying results can be expected. A mixture of urine and feces would probably simulate the natural conditions under which disinfectants are ordinarily used more closely than any of the other forms of organic matter, but the organic content of different specimens of urine and feces is so variable as to practically exclude their use in standardization work. Blood serum is constant in composition, but it is often difficult to obtain, particularly in a sterile condition, and, of course, organic matter must be sterile when it is used.

The results obtained with starch and with egg albumen used as organic matter were, on the whole, unsatisfactory.

Dead broth cultures of the typhoid bacillus and the peptone-gelatin mixture gave rather similar results, but on account of the ease with which the latter can be procured and prepared we have chosen it for use in our work. Peptone and gelatin are fairly constant in composition, and a mixture of the two can be sterilized without altering the composition of either the peptone or the gelatin.

HYGIENIC LABORATORY PHENOL COEFFICIENT

A. Without Organic Matter

Having discussed the necessity for a satisfactory method of standardizing disinfectants and the factors involved in the examination of disinfectants, we present below the method we have devised.

When this method is used for the standardization of disinfectants we recommend that it be referred to as the "Hygienic Laboratory phenol coefficient."

We prefer to use the word "phenol" instead of "carbolic acid" when speaking of the coefficient, especially since certain dealers advertise for sale carbolic acids which vary greatly in the proportion of phenol present.

MEDIA

Standard extract broth is used, both for the culture to be tested and for the subcultures made after exposure to the disinfectant. The broth is made from Liebig's extract of beef and is in exact accordance with the standard methods adopted by the American Public Health Association for water analysis. Ten cubic centimeters of the broth are put into each test-tube. This amount of broth has been found sufficient to avoid any antiseptic action of the disinfectant carried over. It is important that the reaction of the media is just +1.5.

ORGANISM

For the test organism a 24-hour-old broth culture in extract broth of the B. typhosus (Hopkins) is used. Before beginning a test the culture should be carried over every 24 hours on at least 3 successive days. For carrying over the culture one loopful of a 4-millimeters platinum loop is used.

Before being added to the disinfectant the culture is well shaken, filtered through a sterile filter-paper, and placed in the water bath in order that it may reach a temperature of 20°C. before being added to the disinfectant.

TEMPERATURE

A standard temperature of 20°C. has been adopted for all experiments. This temperature is obtained by the use of a specially devised water bath. The culture and dilutions of the disinfectant are brought to this temperature before the beginning of the test.

PROPORTION OF CULTURE TO DISINFECTANT

One-tenth cubic centimeter of the culture is used, added to 5 cc. of the disinfectant dilution. The amount of culture is measured with a pipette graduated in tenths of a cubic centimeter.

INOCULATION LOOPS

For making the transfer of the culture after exposure to the disinfectant a platinum loop 4 millimeters in diameter of 23 United States standard gauge wire is used. We have found it is of advantage to have at least four, and preferably six, loops. In order to save time in flaming the following method was devised:

A block about 3 inches wide, 10 inches high, and 12 inches long, containing four or six grooves, spaced 2 inches apart, is used. Into each of the grooves the platinum loop is laid so that the end of the loops extend about 5 inches beyond the side of the block. The first step in the operation is to sterilize each loop by flaming with a fantail Bunsen burner before beginning the experiment.

When ready to begin the operation the loop farthest from the operator is taken in the right hand and the inoculation made. It is then replaced in the groove with the right hand and the Bunsen burner (fantail) placed under it with the left hand. The next loop is then used, replaced in its groove, and the Bunsen burner placed under it with the left hand, the first loop having been heated to redness while the second loop was in use. This procedure is then continued until all the inoculations have been made. The time required in making the inoculations and in replacing the loop is short, it being found that 15 seconds is ample.

INCUBATION

The subcultures are incubated 48 hours at 37°C., and the results then read off and tabulated.

DILUTIONS

Capacity pipettes for the original dilutions are invariably used for the phenol controls a standard dilution of pure phenol ("Merck's Silver Label") is made and standardized by the U. S. P. method (Koppeschaar) to contain exactly 5 per cent. of pure phenol by weight. From this stock solution the higher dilutions are made fresh each day for that day's test.

For the dilutions of the disinfectant a 5 per cent. solution is made by adding 5 cc. of the disinfectant to 95 cc. of sterile distilled water. A standardized 5 cc. capacity pipette is used for this, and after filling the pipette is wiped off with sterile gauze. The contents of the pipette are then delivered into a cylinder containing 95 cc. of sterile distilled water, and the pipette washed out as clean as possible by aspiration and blowing out the contents of the pipette into the cylinder. The contents of the cylinder are then thoroughly shaken and the dilutions up to 1:500 made from it, using delivery pipettes for measuring. For those disinfectants which do not readily form a 5 per cent. solution we make a 1 per cent. stock solution, and from this make the dilutions greater than 1:100 in accordance with the second table of dilutions. If greater dilutions than 1:500 are to be made, a 1 per cent. solution is made from the 5 per cent. solution, and the higher dilutions made from this.

We have adopted the following scale for making dilutions:

For dilutions up to 1:70, increase or decrease by a difference of 5 (*i.e.*, parts of water).

From 1:70 to 1:160 by a difference of 10.

From 1:160 to 1:200 by a difference of 20.

From 1:200 to 1:400 by a difference of 25.

From 1:400 to 1:900 by a difference of 50.

From 1:900 to 1:1800 by a difference of 100.

From 1:800 to 1:3200 by a difference of 200.

And so on if higher dilutions are necessary.

It is important that the cylinders used for making the dilutions be correctly graduated, as we have found disregard of this factor an important source of error. It is preferable to use standardized cylinders and pipettes, and we recommend that they be used whenever possible. They, of course, should be perfectly clean. For making the dilutions in accordance with the above scheme we have found the following tables of much service:

Table 15 (For Dilutions).—Stock 5 Per Cent. Solution

(5 cc. disinfectant + 95 cc. distilled water = solution A)

		Cc. of A	Cc. of dist. water		Cc. of A	Cc. of dist. water		Cc. of A	Cc. of dist. water
1:20	=	20 +	0	or	10 −	0	or	4 +	0
1:25	=	20 +	5	or	10 +	2½	or	4 +	1
1:30	=	20 +	10	or	10 +	5	or	4 +	2
1:35	=	20 +	15	or	10 +	7½	or	4 +	3
1:40	=	20 +	20	or	10 +	10	or	4 +	4
1:45	=	20 +	25	or	10 +	12½	or	4 +	5
1:50	=	20 +	30	or	10 +	15	or	4 +	6
1:55	=	20 +	35	or	10 +	17½	or	4 +	7
1:60	=	20 +	40	or	10 +	20	or	4 +	8
1:65	=	20 +	45	or	10 +	22½	or	4 +	9
1:70	=	20 +	50	or	10 +	25	or	4 +	10
1:70	=	20 +	50	or	10 +	25	or	4 +	10
1:80	=	20 +	60	or	10 +	30	or	4 +	12
1:90	=	20 +	70	or	10 +	35	or	4 +	14
1:100	=	20 +	80	or	10 +	40	or	4 +	16
1:110	=	20 +	90	or	10 +	45	or	4 +	18
1:120	=	20 +	100	or	10 +	50	or	4 +	20
1:130	=	20 +	110	or	10 +	55	or	4 +	22
1:140	=	20 +	120	or	10 +	60	or	4 +	24
1:150	=	20 +	130	or	10 +	65	or	4 +	26
1:160	=	20 +	140	or	10 +	70	or	4 +	28
1:160	=	20 +	140	or	10 +	70	or	4 +	28
1:180	=	20 +	160	or	10 +	80	or	4 +	32
1:200	=	20 +	180	or	10 +	90	or	4 +	36
1:200	=	20 +	180	or	4 +	36	or	2 +	18
1:225	=	20 +	180	or	4 +	41	or	2 +	20½
1:250	=	20 +	230	or	4 +	46	or	2 −	23
1:275	=	20 +	255	or	4 +	51	or	2 +	25½
1:300	=	20 +	280	or	4 +	56	or	2 +	28
1:325	=	20 +	305	or	4 +	61	or	2 +	30½
1:350	=	20 +	330	or	4 +	66	or	2 +	33
1:375	=	20 +	355	or	4 +	71	or	2 +	35½
1:400	=	20 +	380	or	4 +	76	or	2 +	38
1:450	=	20 +	430	or	4 +	86	or	2 +	43
1:500	=	20 +	480	or	4 +	96	or	2 +	48

Table 16 (For Dilutions).—Stock 1 Per Cent Solution

[1 cc. disinfectant + 99 cc. distilled water = solution B]

		Cc. of A	Cc. of dist. water		Cc. of A	Cc. of dist. water		Cc. of A	Cc. of dist. water
1:100	=	100 +	0	or	10 +	0			
1:110	=	100 +	10	or	10 +	1			
1:120	=	100 +	20	or	10 +	2			
1:130	=	100 +	30	or	10 +	3			
1:140	=	100 +	40	or	10 +	4			
1:150	=	100 +	50	or	10 +	5			
1:160	=	100 +	60	or	10 +	6			
1:160	=	100 +	60	or	10 +	6			
1:180	=	100 +	80	or	10 +	8			
1:200	=	100 +	100	or	10 +	10			
1:200	=	100 +	100	or	10 +	10	or	4 +	4
1:225	=	100 +	125	or	10 +	12½	or	4 +	5
1:250	=	100 +	150	or	10 +	15	or	4 +	6
1:275	=	100 +	175	or	10 +	17½	or	4 +	7
1:300	=	100 +	200	or	10 +	20	or	4 +	8
1:325	=	100 +	225	or	10 +	22½	or	4 +	9
1:350	=	100 +	250	or	10 +	25	or	4 +	10
1:375	=	100 +	275	or	10 +	27½	or	4 +	11
1:400	=	100 +	300	or	10 +	30	or	4 +	12
1:400	=	10 +	30	or	4 +	12	or	2 +	6
1:450	=	10 +	35	or	4 +	14	or	2 +	7
1:500	=	10 +	40	or	4 +	16	or	2 +	8
1:500	=	10 +	45	or	4 +	18	or	2 +	9
1:600	=	10 +	50	or	4 +	20	or	2 +	10
1:650	=	10 +	55	or	4 +	22	or	2 +	11
1:700	=	10 +	60	or	4 +	24	or	2 +	12
1:750	=	10 +	65	or	4 +	26	or	2 +	13
1:800	=	10 +	70	or	4 +	28	or	2 +	14
1:850	=	10 +	75	or	4 +	30	or	2 +	15
1:900	=	10 +	80	or	4 +	32	or	2 +	16
1:900	=	5 +	40	or	4 +	32	or	2 +	16
1:1000	=	5 +	45	or	4 +	36	or	2 +	18
1:1100	=	5 +	50	or	4 +	40	or	2 +	20
1:1200	=	5 +	55	or	4 +	44	or	2 +	22
1:1300	=	5 +	60	or	4 +	48	or	2 +	24
1:1400	=	5 +	65	or	4 +	52	or	2 +	26
1:1500	=	5 +	70	or	4 +	56	or	2 +	28
1:1600	=	5 +	75	or	4 +	60	or	2 +	30
1:1700	=	5 +	80	or	4 +	64	or	2 +	32
1:1800	=	5 +	85	or	4 +	68	or	2 +	34
1:1800	=	5 +	85	or	4 +	68	or	2 +	34
1:2000	=	5 +	95	or	4 +	76	or	2 +	38
1:2200	=	5 +	105	or	4 +	84	or	2 +	42
1:2400	=	5 +	115	or	4 +	92	or	2 +	46
1:2600	=	5 +	125	or	4 +	100	or	2 +	50
1:2800	=	5 +	135	or	4 +	108	or	2 +	54
1:3000	=	5 +	145	or	4 +	116	or	2 +	58
1:3200	=	5 +	155	or	4 +	124	or	2 +	62

SEEDING TUBES

The seeding tubes are glass test-tubes 1 inch in diameter and about 3 inches long, with round bottoms. In order to measure the disinfectant into them they are placed in a suitable wooden stand to receive them. We found it convenient to use a wooden block containing six rows of 15 holes each for the disinfectant to be tested and a separate stand for the phenol controls. The tubes are placed in the stand and each marked with the strength of dilution it is to contain.

Starting with the lowest dilution (*i.e.*, the strongest), the cylinder is shaken, then 5 cc. are measured into the tubes marked to receive that strength, using a 5-cc. delivery pipette. In order to economize glassware, the same pipette is used for measuring out the next dilution, first blowing out as much of the remaining liquid as possible; then drawing a pipetteful of the next dilution to be used and discarding that; then filling the pipette a second time, which is emptied into the seeding tube.

The measuring-out being completed, the tubes are placed in the water bath and allowed to stand a few minutes in order that the disinfectant solution may reach the standard temperature. We have not found it necessary to use cotton plugs in the seeding tubes. They are sterilized in paper-lined wire baskets, with the closed end of the tubes up.

SUBCULTURE TUBE RACKS

Wooden racks, with five rows of 14 holes each, are used for holding the subculture tubes, and as plants are made from each mixture of culture and disinfectant every 2½ minutes up to 15 minutes, six tubes are required for each dilution. Thus, in each rack we have ten rows of six tubes each with two empty cross rows of holes remaining, which are utilized by placing over in the next row each tube as it is planted. This makes it easy to keep run of the tubes that are planted. It is well also always to plant from the seeding tube in a certain hole in the water bath into a certain row of tubes in the rack. This, after a little practice, will help to avoid errors in planting.

METHOD OF CONDUCTING THE TEST

If there are in one experiment more than 10 dilutions of the disinfectant, including the phenol controls, the stronger solutions of the disinfectant and phenol are tested first, as it will not be necessary to plant them after 7½ minutes. The weaker solutions are then immediately done and are then planted every 2½ minutes for 15 minutes.

For keeping the time a stop-watch can be used, but an ordinary watch will serve the same purpose by simply starting on the 2½- or 5-minute periods.

When everything is in readiness the culture is added to the disinfectant solutions with a sterile pipette in quantities of $\frac{1}{10}$ cc. to each dilution.

To add the culture, the seeding tube containing the disinfectant is removed from the water bath with the left hand and slanted at an angle of about 45°, and with the right hand the end of the pipette containing the culture is introduced and lightly touched against the side of the tube where the liquid has run away on account of the slanting. At the proper time the culture is allowed to run into the disinfectant solution, the pipette removed, the tube straightened up, gently shaken three times, and replaced in the water bath. The other tubes are done the same way in succession, and it will be found that 15 seconds is ample time for each tube. By adding the culture to the disinfectant with a pipette touched against the side of the seeding tube, accurate measurements can be made and each tube receive exactly the same amount of "seeding," which is not the case when the culture is added by the "drop."

If 10 tubes are to be inoculated, only a few seconds will remain after inoculating the last tube before a plant from the first tube will have to be made.

The mixing tubes are not removed or disturbed in making the planting except to insert the loop or spoon into them, touch the bottom, withdraw, and then make the plant in broth. Every effort is made to insert and withdraw the loops or spoons in a uniform manner. The loops and spoons are bent to an angle of about 45°, where they are joined to the shank, and therefore are always filled with the mixture when withdrawn from the seeding tubes. After making the plants the loops or spoons are flamed as already described.

After an experiment is finished the date and any necessary details can be marked on one of the broth tubes and the rack placed in the incubator at 37° C. for 48 hours. At the end of this time the results are recorded on a chart specially devised for the purpose.

DETERMINING THE COEFFICIENT

After a large number of experiments we have concluded that the method employed by the Lancet commission, with certain modifications, is the best one for determining the coefficient—*i.e.*, the mean between the strength and time coefficients.

In performing the test, plants are made every 2½ minutes up to and including 15 minutes. To determine the coefficient, the figure representing the degree of dilution of the weakest strength of the disinfectant that kills within 2½ minutes is divided by the figure representing the degree of dilution of the weakest strength of the phenol control that kills within the same time. The same is done for the weakest strength that kills in 15 minutes. The mean of the two is the coefficient. The method of determining the coefficient will be seen in Table 17.

Table 17

Name, "A."
Temperature of medication, 20°C.
Culture used, B. typhosus, 24-hour, extract broth, filtered.
Proportion of culture and disinfectant, 0.1 cc. + 5 cc.

Sample	Dilution	Time culture exposed to action of disinfectant for minutes						Phenol coefficient
		2½	5	7½	10	12½	15	
Phenol	1:80	−	−	−				$\frac{\frac{375}{80}+\frac{650}{110}}{2} = \frac{4.69+5.91}{2} = 5.30$
	1:90	+	−	−	−			
	1:100	+	+	+	−	−	−	
	1:110	+	+	+	+	+	−	
Disinfectant "A"	1:350	−	−	−				
	1:375	−	−	−				
	1:400	+	−	−	−			
	1:425	+	+	−	−	−	−	
	1:450	+	+	−	−	−	−	
	1:500	+	+	−	−	−	−	
	1:550	+	+	+	−	−	−	
	1:600	+	+	+	+	−	−	
	1:650	+	+	+	+	+	−	
	1:700	+	+	+	+	+	+	
	1:750	+	+	+	+	+	+	

B. The Determination of the Coefficient with the Addition of Organic Matter

It may be well to briefly discuss here the meaning or significance of the term "phenol coefficient," particularly when it is determined in the presence of organic matter. In general terms the coefficient of a disinfectant may, for practical purposes, be defined as the figure that represents the ratio of the germicidal power of the disinfectant to the germicidal power of carbolic acid, both having been tested under the same conditions.

Although the germicidal power of carbolic acid is taken as the unit of comparison, it is influenced to a certain extent by conditions, particularly the addition of organic matter, or, in other words, it is not a constant unit. This has to be borne in mind when making a comparison of the relative values of the phenol coefficients of a disinfectant determined with and without the addition of organic matter, respectively. It will readily be seen, for instance, that if the germicidal powers of a disinfectant and of carbolic acid were proportionately reduced by the addition of organic matter the coefficient of the disinfectant would remain unchanged, regardless of whether or not organic matter was used. However, the germicidal power of carbolic acid, like the other pure phenols, is, as compared with most other disinfectants, only slightly affected by the addition of organic matter, and therefore serves as a fairly accurate means of estimating, in the presence of organic matter, the germicidal values of disinfectants in general.

The method of determining the coefficient of disinfectants, with the addition or organic matter, is identical in many respects with the method in which no organic matter is used, the former differing from the latter principally in the strengths of the disinfectant dilutions that have to be prepared and in the preparation and addition of the organic matter to the disinfectant dilutions when performing the test. As the method in which no organic matter is added has already been given in detail, further description here will only consist of the differences between the two methods.

DILUTIONS

In making the dilutions of a disinfectant for determining its coefficient in the presence of organic matter, allowance must be made for the further dilution of the disinfectant when the volume of organic matter is added thereto. For instance, if 1 cc. of organic matter is added to 5 cc. of a 1 per cent. dilution of a disinfectant the percentage strength of the disinfectant is proportionately reduced, it then being about 0.83 per cent.

The volume of organic matter that can be added to the disinfectant dilution is, of course, variable, as is also the volume of disinfectant dilution to which it can be added. Consequently, we have decided rather arbitrarily to use the organic matter by adding 1 cc. of it to 4 cc. of the disinfectant dilution contained in a seeding tube. It will be seen that the strength of the disinfectant is reduced 20 per cent. by the addition of the organic matter and that the dilutions of the disinfectant must be made accordingly. For example, if it is desired to test a 1 per cent. strength of a disinfectant it is necessary to prepare a strength of 1.25 per cent., 4 cc. of which becomes a 1 per cent. strength when 1 cc. of the organic matter is added to it.

We also tried adding 2.5 cc. of the organic matter to 2.5 cc. of the disinfectant dilution, but found it rather difficult and cumbersome to do, particularly when the experiment has to be performed with a number of different strengths of the disinfectant. When using the proportions as just stated, the dilutions of the disinfectant are made double the strengths it is desired to test, thus allowing for the further dilution when an equal volume of organic matter is added thereto.

In using the proportions of 1 cc. of organic matter to 4 cc. of the disinfectant dilution we have found the following tables (18 and 19) of service in preparing the dilutions of the disinfectant.

TABLE 18.—STOCK 5 PER CENT. SOLUTION

[5 cc. of disinfectant + 95 cc. distilled water = solution A]

Strength to be tested	Strength to be made		Cc. of A		Cc. of dist. water		Cc. of A		Cc. of dist. water
1:30	1:24	=	20	+	4	or	10	+	2
1:35	1:28	=	20	+	8	or	10	+	4
1:40	1:32	=	20	+	12	or	10	+	6
1:45	1:36	=	20	+	16	or	10	+	8
1:50	1:40	=	20	+	20	or	10	+	10
1:55	1:44	=	20	+	24	or	10	+	12
1:60	1:48	=	20	+	28	or	10	+	14
1:65	1:52	=	20	+	32	or	10	+	16
1:70	1:56	=	20	+	36	or	10	+	18
1:70	1:56	=	10	+	18	or	5	+	9

TABLE 18.—STOCK 5 PER CENT. SOLUTION.—(*Continued*)

Strength to be tested	Strength to be made		Cc. of A	Cc. of dist. water		Cc. of A	Cc. of dist. water
1:80	1:64	=	10 +	22	or	5 +	11
1:90	1:72	=	10 +	26	or	5 +	13
1:100	1:80	=	10 +	30	or	5 +	15
1:110	1:88	=	10 +	34	or	5 +	17
1:120	1:96	=	10 +	38	or	5 +	19
1:130	1:104	=	10 +	42	or	5 +	21
1:140	1:112	=	10 +	46	or	5 +	23
1:150	1:120	=	10 +	50	or	5 +	25
1:160	1:128	=	10 +	54	or	5 +	27
1:160	1:128	=	10 +	54	or	5 +	27
1:180	1:144	=	10 +	62	or	5 +	31
1:200	1:160	=	10 +	70	or	5 +	35
1:200	1:160	=	5 +	35	or	2 +	14
1:225	1:180	=	5 +	40	or	2 +	16
1:250	1:200	=	5 +	45	or	2 +	18
1:275	1:220	=	5 +	50	or	2 +	20
1:300	1:240	=	5 +	55	or	2 +	22
1:325	1:260	=	5 +	60	or	2 +	24
1:350	1:280	=	5 +	65	or	2 +	26
1:375	1:300	=	5 +	70	or	2 +	28
1:400	1:320	=	5 +	75	or	2 +	30
1:400	1:320	=	5 +	75	or	2 +	30
1:450	1:360	=	5 +	80	or	2 +	32
1:500	1:400	=	5 +	85	or	2 +	34
1:550	1:440	=	5 +	90	or	2 +	36
1:600	1:480	=	5 +	95	or	2 +	38
1:650	1:520	=	5 +	100	or	2 +	40

TABLE 19.—STOCK 1 PER CENT. SOLUTION

[1 cc. disinfectant + 99 cc. distilled water = solution B]

Strength to be tested	Strength to be made	Cc. of B	Cc. of dist. water
1:500	1:400	5 +	15
1:550	1:440	5 +	17
1:600	1:480	5 +	19
1:650	1:520	5 +	21
1:700	1:560	5 +	23
1:750	1:600	5 +	25
1:800	1:600	5 +	27
1:850	1:680	5 +	29
1:900	1:720	5 +	31
1:900	1:720	5 +	31
1:1000	1:800	5 +	35
1:1100	1:880	5 +	39
1:1200	1:960	5 +	43
1:1300	1:1040	5 +	47
1:1400	1:1120	5 +	51
1:1500	1:1200	5 +	55
1:1600	1:1280	5 +	59
1:1700	1:1360	5 +	63
1:1800	1:1440	5 +	67

It will be seen that by preparing the dilutions of the disinfectant according to the strengths shown in the second column of Table 18, and then adding 1 cc. of organic matter to 4 cc. of the dilutions, the final dilutions of the disinfectant become as represented in the first column of the table given above.

PREPARATION AND USE OF THE PEPTONE-GELATIN ORGANIC MATTER

As already stated, we prefer to use a mixture of peptone and gelatin dissolved in distilled water. It is prepared from Witte's petpone (siccum) and "Best French Gold Label" gelatin. The stock preparation is made to contain 10 per cent. peptone and 5 per cent. gelatin. Proportionate amounts of peptone and gelatin respectively, are weighed out and liquefied separately in small quantities of water by means of heat. They are then mixed in a graduate and sufficient water added thereto to make a mixture containing 10 per cent. of peptone and 5 per cent. of gelatin. It is then placed in bottles of appropriate size and sterilized on 3 successive days. When the mixture has become cold it will be observed that some of the peptone settles to the bottom of the bottles as a flocculent deposit. Consequently, the bottle should be shaken before using it. The stock preparation containing 5 per cent. gelatin becomes semi-solid if it is kept in the cold room at a temperature of 16°C. However, by warming it until it becomes perfectly liquefied and then not allowing it to go below the temperature of 20°C. we found that it remains liquid and is easily measured in the pipette.

By adding 1 cc. of the stock preparation of 10 per cent. peptone and 5 per cent. gelatin to 4 cc. of the disinfectant dilutions a resulting mixture is obtained containing 2 per cent. of peptone and 1 per cent. of gelatin, or a total of 3 per cent. of organic matter.

THE METHOD OF CONDUCTING THE TEST

Four cc. of each disinfectant dilution, including the phenol controls, are accurately measured into the seeding tubes and placed in the water bath. For reasons that will be obvious later, it is difficult to handle more than nine dilutions at one time in making a test. The broth culture of B. typhosus is filtered and placed in the water bath. The desired quantity of stock peptone-gelatin mixture is measured into a large test-tube or flask and also placed in the water bath. It is necessary to have a slight excess of the organic matter, so that there will be no trouble in getting the 10-cc. pipette full when adding the organic matter and culture to the disinfectant. Thus, for nine tubes we usually measure out 15 cc. of the organic matter When the disinfectant dilutions, the typhoid culture, and the organic matter have reached the temperature of the water bath (20°C.) 1.5 cc. of the typhoid culture is added to the 15 cc. of organic matter and thoroughly mixed by means of a 10-cc. pipette. With the same pipette the seeding tubes containing the disinfectant dilutions then have added to them, successively every 15 seconds, 1.1 cc. of the mixture of the organic matter and typhoid culture. The technique of shaking, planting, etc., is then as has already been described.

From the above it will be seen that each seeding tube, as the test is conducted, contains 4 cc. of the disinfectant dilution plus 1 cc. of organic matter, plus 0.1 cc. of typhoid culture, and that the quantity of organic matter represented is 3 per cent.

In computing the strengths of the disinfectant and the percentage of organic matter present in the seeding tubes when the test is made, we have disregarded the slight change resulting from the addition of the 0.1 cc. of typhoid culture to each tube. By so doing the experiment is very much simplified, as it would be very difficult to prepare the dilutions of the disinfectant on the basis of allowing for so slight a change as is caused by the addition of 0.1 cc. of liquid.

CHAPTER V

DIAGNOSIS

A bare knowledge of the morphology and biology of pathogenic bacteria is of little value in diagnosis unless we also know how, when and where to search for a them in disease. Some of this information has been imparted in the previous chapters, here an attempt will be made to add additional information that may serve in attempts to disclose the offending organism present in infectious diseases.

DISEASES OF THE SKIN

Some skin diseases are caused by bacteria and others are not. Of the organisms which attack the skin the staphylococcus is probably the most frequent offender. Since the staphylococcus albus is practically always present upon the skin in health and disease, we cannot with assurance look upon it as an etiological factor when obtained in smears or cultures taken from skin lesions, except under the following conditions:

1. When possible contamination by contact with the surface of the skin of the operator or of the patient has been precluded.

2. When the organism has been found in pure culture after attempts to discover other organisms, especially the acne bacillus, have failed.

ACNE

Acne may be caused by the acne bacillus or the staphylococcus. The staphylococcus is found in slides and aerobic cultures made from the core of acne lesions in practically all cases, whether due to the staphylococcus or the acne bacillus.

The acne bacillus is an obligate anaerobe, to obtain it in pure culture from an acne lesion proceed as follows: Wash the skin surrounding the pimple with soap and water and alcohol, remove the top of the pimple with a sterile needle, obtain the lower half of the core with a sterile platinum loop (avoiding contact of either loop or removed material with the patient's skin or any object), plant slant tubes of agar, blood serum and a tube of bouillon and incubate anaerobically at 37°C. for 10 days (by incubating for 10 days the acne bacillus will outgrow staphylococci that are present). After planting culture media obtain a core as before and smear in a thin film on cover glasses, gently heat until dry and stain one with methylene blue, another by Gram's method. The acne bacillus is somewhat similar in morphology to the diphtheria bacillus and is Gram positive.

FURUNCULOSIS AND CARBUNCLES

Furunculosis and carbuncles are commonly caused by staphylococci, sometimes by streptococci, rarely by other organisms. To make a bacteriological diagnosis obtain material as when examining pimples, plant slant tubes of agar and blood serum and incubate aerobically at 37°C. for 24 to 48 hours. Also make slides, stain with methylene blue and by Gram's method.

PHLEGMONA DIFFUSA

After cleaning the surface where the superficial skin is unbroken, and overlying pus or serum exudate, incise, remove several loops full of pus or serum and plant on agar and blood serum and incubate at 37°C. for 24 to 48 hours. Make slides for microscopic examination.

ERYSIPELAS

The streptococcus is looked upon as the sole cause of erysipelas, not every attempt to obtain it from the skin lesions succeeds. Clean the surface and attempt to withdraw fluid from the deep layer of the skin and subcutaneous tissues. Plant it on agar and blood serum and incubate at 37°C. for 24 hours. Make slides.

Search for enlarged glands adjacent to the diseased area, if found, massage, clean overlying skin aspirate some of the contents with a syringe. Culture and make slides from material so obtained.

MALIGNANT PUSTULE

Malignant pustule is caused by the anthrax bacillus. Clean the surrounding skin and overlying tissue, incise and obtain some serum. Plant on agar and blood serum and make slides for microscopic examination.

Withdraw urine with catheter, centrifuge and make cultures and slides from the sediment.

Withdraw 2 cc. of blood from a vein with a sterile syringe, smear several drops over the surface of each of several tubes of agar and blood serum and incubate at 37°C. for 24 to 72 hours. Make smears from the blood and stain for microscopic examination. The anthrax bacillus will only be found in the blood and urine when the disease has passed beyond the initial or local lesion on the skin and has entered the circulation.

FARCY (GLANDERS)

Glanders, or Farcy, as the superficial form of the disease is known, is caused by the bacillus mallei, other organisms, as staphylococci frequently contaminate superficial lesions, especially if open and exposed to the air.

Obtain pus or scraping from a lesion and plant on glycerin agar, blood serum and potato and incubate aerobically at 37°C. for 24 to 72 hours, also make slides and stain.

PURPURA HEMORRHAGICA

Some, probably not all cases of this disease, are of bacterial origin. Cultures on blood-agar, ascitic fluid agar and blood serum should be made from the lesions, venous blood and any enlarged glands that may be found.

IMPETIGO CONTAGIOSA

Impetigo contagiosa may be caused by pathogenic cocci. Observing the necessary precautions to avoid contamination with saprophytic organisms present upon the overlying crusts and contiguous skin, cultures should be made from the lesions upon agar and blood serum.

LUPUS

Lupus, tuberculosis of the skin, is diagnosticated bacteriologically by examining scrapings from the lesions microscopically for tubercle bacilli, by excising a portion of the diseased skin and after proper treatment of it examining microscopically both for tubercle bacilli and typical histological changes, by injecting macerated scrapings from the lesions into guinea-pigs and rabbits and by subjecting the patient to the tuberculin test. Not all these procedures are necessary in every case. The multiplicity of methods of examination is demanded because one or several may fail in a particular case.

FRAMBŒSIA

Frambœsia or yaws is a non-veneral skin disease, similar to some of the superficial manifestations of syphilis. It is caused by a spirochæte indistinguishable from that of syphilis. The disease is diagnosticated by pinching up one of the lesions, nicking the skin with a sharp knife (avoid producing hemorrhage), and collecting the serum in a capillary tube.

The serum is spread in a thin film on cover glasses, stained according to the methods given for treponema pallidum, and examined. Yaws is differentiated from syphilis microscopically by the larger number of spirochæte found in every lesion of yaws than in syphilis.

SYPHILIS

In the first stage of syphilis a single, indurated ulcer develops at the site of infection. More or less generalized macular and papular rashes and ulcers occur from time to time during the second stage of the disease.

The treponema may be found in the eruptions of the second and third stages of the disease by following the technique as given for diagnosis of yaws. It is better, however, to make the diagnosis by the Wassermann test.

In the primary stage of the disease, the Wassermann test is frequently negative, especially early, bacteriological diagnosis must then be made by finding treponema in the chancre or enlarged glands. After the chancre has been free from germicides and disinfectants for 24 hours, and covered with a sterile dressing, the dressing is removed, the ulcer washed with sterile salt solution, and avoiding the production of hemorrhage, serum or serum and scrapings is obtained from the floor of the ulcer with a capillary tube. If adjacent glands are enlarged, some of the contents of these is removed with a syringe. This material is examined unstained with the dark-field microscope, or stained, as described in the chapter on treponema pallidum.

MADURA FOOT AND ACTINOMYCES

In these infections the pus is searched for granules and these are crushed between a slide and a cover glass and examined microscopically, unstained and after staining. When granules are not found, the pus is spread on slides and examined microscopically before and after staining.

FAVUS, RING WORM, PITYRIASIS VERSICOLOR, ERYTHRASMA AND BLASTOMYCETIC DERMATITIS

Bacteriological diagnosis in the diseases caused by the higher forms of bacteria is usually based upon microscopic examination alone, cultures may be made for more accurate classification. In the above-named diseases, the method of procedure is the same, some hairs, portions of the crust or scab and scrapings from the border line between the diseased and healthy skin should be obtained and examined separately. A number of slides should be prepared and examined in every case. The material to be examined, whether hair, crust or scrapings, should be placed in a drop of 20 to 40 per cent. potassium hydrate solution and a cover glass dropped upon it. The slides are then allowed to stand at room temperature for several minutes. Then some are examined without heating, others after gently warming for several minutes and others after heating until steam arises.

In every case where the microscopical examination and other findings do not disclose the exact nature of the offending organisms, cultures should be made.

LEPROSY

In every suspected case of leprosy the anterior nares should be examined whether lesions appear there or not; a sterile cotton swab is passed over the mucous membrane and then rubbed on a slide. Skin ulcers should be cleansed to free them of contamination and slides made from scrapings removed from the floor of the ulcer.

These slides should be stained as when examining for tubercle bacilli.

DISEASES OF THE EYE

The lids are subject to all of the infectious diseases that affect the skin and the technique of bacteriological diagnosis is the same as in similar diseases of the skin of other parts of the body.

The lachrymal apparatus is more frequently attacked by the pyogenic organisms than by other bacteria. When a bacteriological diagnosis of an infection of any part of the lachrymal apparatus is desired, the affected and contiguous parts should be thoroughly washed out with sterile salt solution and covered with a sterile dressing. About 12 hours later the dressing is removed, a sterile loop or cotton swab is passed over the affected membrane and then drawn over the surface of slant tubes of agar and blood-serum media the tubes are incubated at 37°C. for 18 to 72 hours. Slides should also be prepared.

CONJUNCTIVA

In addition to acute catarrhal, contagious conjunctivitis or pink-eye, caused by the Koch-Weeks bacillus and ophthalmia neonatorum caused by gonococcus, infections of the conjunctiva may be caused by a variety of organisms. After cleansing and covering with a sterile dressing for a number of hours, slides should be made and stained with methylene blue and by Gram's method, cultures should be made on appropriate media. The material for examination is obtained by passing a sterile cotton swab over the affected part.

SYPHILIS AND TUBERCULOSIS OF THE EYE

Chancres, though rarely observed in such localities, do occur on the lids and conjunctiva, suspicious ulcers should be examined for treponema pallidum and a Wassermann test made.

Secondary, tertiary and congenital syphilis frequently cause inflammation of the cornea, sclera, iris, ciliary body, choroid, retina and optic nerve, occasionally tuberculosis does the same.

In suspected cases a Wassermann test should be made and if negative a tuberculin test is made.

DISEASES OF THE EAR

The auricle and external auditory canal are subject to all the infections that affect the skin, but they are much less frequently attacked. The technique of bacteriological diagnosis of infections of those parts is the same as in similar infections of the skin.

The diphtheria bacillus sometimes infects the external auditory canal, this is most apt to occur when the throat is also involved and technique for examination is the same as when examining the throat for diphtheria bacilli.

OTITIS MEDIA

Nearly all infections of the middle ear follow, or occur as extensions of, nasopharyngeal infections. When they progress to suppuration and discharge through the external auditory canal secondary infection from the air frequently results.

Bacteriological diagnosis is usually called for when the disease has progressed to suppuration and pus is discharging through the external meatus. In such cases the ear is thoroughly cleansed by syringing with sterile salt solution, a plug of sterile cotton is then placed in the external meatus, 6 to 24 hours later the auricle is asepticized, the plug removed, and a sterile cotton swab is passed into the external auditory canal to collect the exudate. Slides and cultures on agar, ascitic agar and blood serum are made. The organisms most frequently found as the cause of otitis media are the following: staphylococci, bacillus pyocyaneus, streptococci, pneumococcus, bacillus ozena, diphtheria bacillus and bacillus of influenza.

MASTOIDITIS

Mastoid infections are usually the result of extension from the middle ear.

DISEASES OF THE NOSE

Primary, secondary and tertiary lesions of syphilis occur occasionally in the nose and are diagnosticated by examination of lesions for treponema pallidum and chiefly by the Wassermann test.

Diphtheria bacilli may be present in the nose and not elsewhere, in persons not suffering with diphtheria carriers. Nasal diphtheria may occur without the throat being involved; this is rare.

Chronic rhinitis, chronic nasal catarrh, may be caused by staphylococci, the pneumococcus, bacillus of rhinoscleroma, bacillus of ozena and less frequently micrococcus catarrhalis, meningococcus and other organisms. Any of these organisms may be present at times upon normal nasal mucosa.

To make a bacteriological examination pass a sterile cotton swab into each nostril, brush it over as much of the membrane as possible, withdraw and make surface plants upon agar, ascitic fluid agar and blood serum. Also prepare slides for microscopic examination.

When possible it is well to make a second set of cultures and slides after preliminary treatment of the nose as follows:

Thoroughly cleanse the nose, wash out with sterile salt solution and place a plug of cotton in each nostril so as to filter the air without impairing inspiration, keep the patient in a room free of gas for 12 hours and then obtain material for culture and slides.

DISEASES OF THE LUNGS

Bacteriological diagnosis of diseases of the lungs (other than syphilis which is rare) is based upon the microscopic examination of the sputum, occasionally supplemented by cultures from sputum and inoculation of the sputum into rabbits and guinea-pigs.

The technique for obtaining sputum and examining it has already been considered in the chapters on the pneumococcus and tubercle bacillus. Here it is only necessary to add that pneumonia is not a specific disease. While the pneumococcus is responsible for most cases of acute pneumonia, still, a large number of cases are caused by other organisms, staphylococci, bacillus of influenza, streptococci and the tubercle bacillus. In acute pneumonia, caused by the pneumococcus, that organism is usually found to predominate in the sputum some time during the disease, in nearly all cases, yet it must be remembered that occasionally it does not, especially early in the disease.

It is most important to remember there is a group of cases which start as, run the typical course of, and are clinically diagnosticated as acute lobar pneumonia caused by the pneumococcus; that bacteriological examination of the sputum prior to crisis shows a preponderance of pneumococci in most cases and seldom shows tubercle bacilli; that after crisis recovery does not follow, cough and expectoration continues, resolution is incomplete, the sputum may still show a preponderance of pneumococci, but usually many organisms are present, tubercle bacilli are not discovered for weeks or months; prior to their discovery it becomes obvious that the case is one of pulmonary tuberculosis. In all such cases where resolution and convalescence are retarded following crisis a tuberculin test should be made.

DISEASES OF THE GASTRO-INTESTINAL TRACT AND CONTINUOUS FEVERS

The signs, symptoms and clinical course of typhoid fever, paratyphoid fever, food-poisoning, tuberculosis, syphilis, gonococcus, staphylococcus and streptococcus infections and malaria are at times indistinguishable, except by careful bacteriological studies.

In typhoid, paratyphoid, colon bacillus and other infections caused by members of the typhoid-colon group, during the first week of the disease the offending organism can always be isolated from the blood by culture. Later than the second week the offending organism or its specific agglutinin can be found in the blood in nearly all cases. These tests should be made in all such cases, and when negative, foci of infection with pyogenic cocci should be sought for; blood cultures for staphylococci, streptococci and pneumococci should be made. If these fail to establish the diagnosis the Wassermann and possibly the gonococcus complement fixation tests should be made.

In suspected cases of cholera and bacillary dysentery the feces should be examined by culture for the offending organism and the blood serum for specific agglutinin and lysin.

Huston and McCloy, The Lancet, vol. ii, No. 15, Oct. 7, 1916, have reported the isolation of an organism, called "enterococcus" from the feces of patients with a cryptogenic illness, which they believe is due to this organism. They believe the "enterococcus" is not of the normal bacterial flora of the intestine and suggest the advisability of attempts to isolate possible causative organisms from the feces in all cases of cryptogenic gastro-intestinal disease.

DISEASES OF THE GENITO-URINARY TRACT

ULCERS OF THE EXTERNAL GENITALIA

Ulcers of the external genitalia may be due to physical, chemical or vital causes. Any of the organisms that produce ulceration of the skin elsewhere may do so here. As a matter of fact, nearly all the ulcers of the external genital organs that are brought to the bacteriologist for diagnosis are chancres or chancroids, the result of venery, and the problem is to discover whether they are caused by the treponema pallidum or other organisms.

The technique of examination is as follows: Thoroughly cleanse the ulcer and surrounding parts and cover with a sterile dressing, without any germicide or antiseptic; after this dressing has been on for 12 to 24 hours remove it and obtain some serum, also scrapings from the floor of the ulcer; examine these specimens unstained, microscopically, using the dark-field microscope; if treponema pallidum is observed the diagnosis is established; if not observed, proceed as follows: obtain serum and scrapings from the ulcer, spread on slides or cover glasses, dry without heating and stain—some with methylene blue, others by Gram's method and others by Giemsa's method for treponema pallidum; if desired, cultures may be made on blood-agar, ascitic agar and inoculation into a rabbit's testicle.

The treponema pallidum is present in chancres, either in pure culture or together with other organisms.

Chancre and chancroid may exist together, in which case the organisms of both conditions will be found.

Ducrey's bacillus (bacillus of soft sore) is considered the specific cause of chancroid. Other organisms are usually found with it—staphylococci, gonococci or micrococcus tetragenus.

Ducrey's bacillus is a short rod or oval, 0.5 μ by 2.0 μ, has round ends, is non-motile, is arranged singly and in chains, occurs within pus cells and outside of them, stains readily with all the usual anilin dyes and is Gram negative. It does not stain uniformly, the poles stain deep, the intervening portion faintly or not at all.

Ducrey's bacillus does not grow on ordinary media. On blood-agar incubated aerobically at 31°C., round, raised, pin-head-sized, opaque, grayish colonies develop in 24 to 48 hours.

ACUTE URETHRITIS AND VAGINITIS

Acute urethritis or vaginitis may be caused by staphylococci or gonococci, rarely by other organisms. Microscopic examination of smears made from the discharge and stained by methylene blue and by Gram's method will establish the diagnosis in nearly all cases.

CHRONIC URETHRITIS AND VAGINITIS

Chronic urethritis and vaginitis may be due to a single organism, usually several are present. When due to a single organism it may be impossible to find it except following an unusual irritation or hypersecretion such as may be produced by drinking beer, massage of the prostate or sexual excitement. If spreads and cultures fail to show the offending organism, they should be again made after instituting irritation or hypersecretion. Cultures are made by picking up some of the discharge and planting it on the surface of Loeffler's blood-serum medium and on neutral ascitic agar. Tubes containing no free moisture must be used and it is best to plant several so that some may be incubated with reduced oxygen tension and others aerobically at 37°C.

When chronic urethritis or vaginitis is due to several organisms there is usually sufficient discharge to permit of diagnosis by microscopic examination of smears and by culture at any time. In such cases gonococci, staphylococci, pseudodiphtheria bacilli, the colon bacillus or the diphtheria bacillus may be present.

INFECTION OF THE URINARY BLADDER

Bacteriologically, infection of the urinary bladder can only be determined with accuracy by microscopic and cultural examination of urine obtained through a sterile catheter under conditions which preclude the possibility of bacteria entering the urine outside the bladder.

INFECTION OF THE KIDNEYS

Bacteriologically, renal infection can only be detected by microscopic and cultural examination of urine obtained through ureteral catheter.

Any of the organisms that may infect the urethra can infect the bladder or kidney. The colon bacillus is probably the most frequent invader of the bladder and kidney.

When urine is to be examined for bacteria other than tubercle bacilli the examination should be made immediately after removal from the body. The technique described on pages 116 and 117 should be employed; in addition, plants on plain agar, or better, on blood-agar, should also be made.

For the tubercle bacilli see page 98.

There is a wide difference of opinion as to the significance of tubercle bacilli found in urine, also as to whether or not acid-fast organisms found in urine may properly be considered tubercle bacilli.

The results of extensive laboratory studies conducted by Prof. R. C. Rosenberger and the coördinated clinical and laboratory findings of Dr. Charles Bonney and the writer may be summarized as follows:

First, acid-fast bacteria (which are similar to, but which are not tubercle or leprosy bacilli, such as occur at times in sputum, smegma, feces, urine and water), occasionally resist decolorization and remain red after immersion in Pappenheim's solution for 2 minutes. Rarely, indeed, less than once in a hundred times, do such organisms remain red after twenty minutes' immersion in Pappenheim's solution.

Second, we have never found red bacilli in urine from clinically non-tuberculous individuals when (*a*) the external genital organs are carefully cleansed prior to micturition or catheterization, (*b*) the urine is collected in sterile containers and protected from extra corporeal contamination, and (*c*) the smears made for microscopic examination are immersed in Pappenheim's solution for 20 minutes or more, after carbol fuchsin staining.

Third, whenever we have found red bacilli, identified as tubercle bacilli, in urine, there has been at the time, or later, conclusive evidence of active tuberculosis of one or both kidneys, usually, but not always, associated with distinct physical signs of latent or active pulmonary tuberculosis.

ARTHRITIS

Most cases of arthritis are due to infection. It is generally believed that the atria of infection in most cases is through the tonsils or teeth. When tonsillar or mouth infection and arthritis coexist, the cause of the arthritis is sometimes sought for in cultures obtained from the tonsils; such conclusions are quite as apt to be erroneous as correct.

The offending organism can sometimes, but not always, be found in fluid or pus aspirated from the diseased joint, and an attempt to do so should be made when bacteriological diagnosis is attempted.

In nearly all cases of arthritis caused by staphylococci, streptococci or the pneumococcus, careful search will reveal the presence of enlarged glands adjacent to the affected joint. The offending organism can be found in these if the following procedure is carried out so carefully that contamination of the gland with extraneous bacteria is precluded:

Asepticize the skin overlying the enlarged gland, incise the skin and remove the gland, place the gland in a sterile mortar and emulsify it with sterile water, plant on slant tubes of agar, blood-agar and ascitic fluid agar, also make deep stab cultures in tubes containing agar and blood-agar to the depth of 6 inches. Incubate aerobically at 37°C.

Inject the remaining portion of gland emulsion into the peritoneal cavity of a guinea-pig.

It is to be remembered that a large number of "rheumatic" manifestations, muscular as well as arthritic, are due to syphilis and that the Wassermann test is positive in 75 per cent. or more of these syphilitic cases.

The gonococcus complement fixation test will often establish the diagnosis, in cases of gonococcus arthritis, when other means fail.

In tubercular arthritis the tubercle bacillus is practically never found in slides or cultures made from the fluid or pus; animal inoculations sometimes give positive results. In suspected cases of tubercular arthritis, when other means of diagnosis fail, when there is no contraindication, a tuberculin test should be made.

CHAPTER VI

BACTERIAL VACCINES

Bacterial vaccines with few exceptions as noted are suspensions or solutions of attenuated or dead bacteria, or bacterial products, in normal salt solution.

Stock vaccines are those made from cultures kept in the laboratory.

Autogenous vaccines are those made from cultures of the offending organism or organisms obtained from the infected person and used in the treatment of that patient.

Polyvalent vaccines are those made from various strains of the same organism obtained from different sources. Mixed vaccines are those containing two or more different species of bacteria.

Some vaccines are prepared, standardized, sterilized and administered by special methods. In general, most vaccines are made from 24-hour-old cultures on the surface of solid media. The medium used depends upon the requirements of the organism one desires to make the vaccine with. Those that grow well on agar are planted on agar; those that grow best on blood-agar or ascitic agar are planted on them. Young cultures are most desirable but if growth is not apparent in 24 hours incubation is extended to 48 or occasionally 72 hours. The bacteria are procured by pouring sterile normal salt solution on the media and shaking or scraping until the bacteria are suspended in the salt solution.

The salt solution suspension of bacteria is then poured into a sterile tube or flask and shaken to break up clumps.

The number of bacteria per cubic centimeter is estimated by mixing equal-sized drops of blood and bacterial suspension on a glass slide, spreading in a thin, even film, staining with any blood stain and examining under an oil immersion lens. A satisfactory count can only be made, when the shaking of the bacterial suspension has been sufficient to break up clumps and evenly distribute the individual organisms; when the mixture of blood and bacterial suspension has been done so rapidly that the film is spread before clotting occurs; when the film is thin enough to present fields containing bacteria and red cells not too numerous to count when examined through the oil immersion lens. The bacteria and red cells are counted in each field until a total of 500 red cells has been counted; the number of bacteria counted is totaled. Multiply the number of bacteria counted by 10,000,000. This gives the number of bacteria in each cubic centimeter of the vaccine.

This computation is based on the fact that normal blood contains 5,000,000,000 red cells per cubic centimeter.

After the number of bacteria per cubic centimeter has been determined the suspension of bacteria is further diluted by the addition of sterile normal salt solution so as to bring the bacterial content to 1,000,000,000 per cubic centimeter.

Vaccines are sterilized by heat in a water bath at the minimum temperature and time of exposure that will kill the organism.

After heating, several loopsful of the vaccine are planted on culture media and incubated to prove sterility.

When the vaccine has been sterilized, 0.10 to 0.25 per cent. of tricresol is added and the vaccine sealed in appropriate sterile containers.

The most important vaccines made, according to the technique just described, will be mentioned, also the media upon which they may be cultivated, temperatures and time of exposure required to sterilize and purpose for which they are employed:

TYPHOID VACCINE

All strains of the typhoid bacillus do not possess equal immunizing properties. One having proved immunizing power is used. It is planted on plain agar. The bacterial suspension is sterilized in a water bath at 56°C. to 60°C. for ½ hour.

Typhoid vaccine is used to produce immunity, to combat sequelæ of typhoid fever and sometimes in the treatment of typhoid fever.

Dose.—The average immunizing dosage consists of three injections at intervals of 10 days; the first 500,000,000, second 1,000,000,000 and third 1,000,000,000 organisms. In the treatment of typhoid fever and sequelæ the dose varies from 500,000 to 1,000,000,000 at intervals between injections from 2 to 20 days.

Vaccines of the various paratyphoid bacilli, the bacillus coli, the various dysentery bacilli and the spirillum of cholera are made in the same way, have the same dosage and are employed principally to immunize against infection and in the treatment of sequelæ, occasional and with less success in the treatment of subacute or prolonged cases of these diseases.

GONOCOCCUS VACCINE

Gonococci are cultured on media composed of about 6 parts glycerin agar and 4 parts of blood, blood serum, ascitic fluid, or egg. The vaccine is sterilized in a water bath at 55°C. to 60°C. for from 10 to 30 minutes. Gonococcus vaccine does not confer immunity; it is sometimes of value in acute urethritis, but seems most effective in subacute and chronic infections and sequelæ of gonorrhœa.

The average initial dose for children is 5,000,000, for adults 25,000,000. Reactions are to be avoided.

Meningococcus vaccine is made in the same way but is seldom used and there is not much evidence in regard to its indications or value.

STAPHYLOCOCCUS VACCINE

When making a stock vaccine a polyvalent one should be made. If it is to be a staphylococcus aureus vaccine, then pure cultures of staphylococcus aureus should be obtained from as many different sources as possible—from the blood in cases of staphylococcus aureus bacteremia, from infected wounds, from abscesses, from the throat in cases of staphylococcus, tonsillitis, etc.

These various strains are cultivated on plain agar, suspended in salt solution, mixed and sterilized in the water bath at 60°C. to 80°C. from 30 minutes to 1 hour.

Staphylococcus vaccines are employed to fortify natural immunity and to combat staphylococcus infections. The average initial dose is from 50,000,000 to 100,000,000.

Staphylococcus albus and citrus vaccines are made in the same way; the same is true of streptococcus and pneumococcus except that these two are best cultivated on blood-agar. The average dose of all is the same.

Vaccines of any of these cocci seldom produce any marked exaltation of immunity when given to healthy persons; they do not as a rule conspicuously modify or curtail acute infections, their chief value is in the treatment of subacute and chronic infections.

There are innumerable strains of staphylococci that are indistinguishable but differ in that a vaccine made from one does not stimulate antibody formation or immunity to the others. The same is true of streptococci and pneumococci. Therefore, autogenous vaccines are very much superior to stock vaccines in the treatment of such infections.

SENSITIZED VACCINES

Sensitized vaccines are prepared by cultivating the organism on its appropriate medium, washing off the growth with sterile salt solution, shaking the suspension to break up clumps and counting the number of bacteria per cubic centimeter as previously described. The suspension is then centrifugalized at high speed and when the bacteria have been precipitated the supernatant salt solution is pipetted off and discarded. The bacteria are mixed with the serum of an animal immunized against the same organism. This mixture is incubated at 37°C. for 24 hours and then centrifugalized until bacteria precipitate. The serum is syphoned off and discarded. The bacteria are washed in several changes of normal salt solution to rid them of serum. They are mixed with sufficient salt solution to make a suspension containing the desired amount of bacteria per cubic centimeter; and are then sterilized in a water bath at 55°C. to 60°C. for ½ hour.

This sensitization, when successfully carried out, is said to prevent or minimize undesirable reactions following the injection of the vaccine, to prevent the occurrence of a negative phase to permit the administration of larger doses and repetition of doses at shorter intervals and to produce a higher degree of immunity than it is possible to secure with non-sensitized vaccines.

TUBERCULIN

Tuberculin is used by some in the treatment and in the diagnosis of tuberculosis.

From among the various tuberculins, the selection of one for use in the treatment of a particular case is determined by the signs, symptoms, localization, extent and progress of the disease and by the fancies of the person using the tuberculin.

Tuberculin is of greatest value as a diagnostic agent.

Koch's Old Tuberculin.—(Tuberculinum original alt) "T. O. A." or "O. T." is made by planting the tubercle bacillus in a flask of glycerin broth, incubating at 37°C. for 8 weeks. It is then placed in steam sterilizer for 1 hour or autoclave at 15 pounds pressure for ¼ hour and evaporated on a water bath at 80°C. to $\frac{1}{10}$ its original volume. The bacteria are removed by filtration, first through paper and then through a Berkefeld filter, the filtrate, which is the tuberculin, is put in containers and sterilized in steam sterilizer, 1 hour each day for 3 successive days, or in the autoclave at 15 pounds pressure for 20 minutes.

Deny's Bouillon Filtrate.—(Bouillon Filtrate) "B. F." is prepared the same as O. T. except that it is not heated at any time during its preparation. It is evaporated to one-tenth its original volume at room temperature *in vacuo* over sulphuric acid.

Koch's Bacillen Emulsion.—"B. E." cultures are made as for the preparation of O. T., the pellicle of bacteria is caught on cheesecloth as the culture is poured on it, they are freed of broth by pouring over them first salt solution, then water. The bacteria are placed in dishes and desiccated *in vacuo* over sulphuric acid and weighed, then ground in a porcelain ball mill for about 6 weeks; 100 cc. of salt solution for each gram of bacteria is then put in the mill and grinding continued for 2 days. The emulsion is allowed to stand at rest for several days so that coarser particles precipitate, the supernatant fluid is then collected, mixed with 50 per cent. of glycerin and standardized to contain 5 mg. of solids in each cubic centimeter.

Tuberculin Residue "T. R." ("New tuberculin").—Bacilli are grown and recovered from the culture, dried, weighed and powdered, the same as when proceeding to make B. E. When sufficiently comminuted, 100 cc. of sterile distilled water is added to each gram of dried bacteria, and the mass again ground for 24 hours. The emulsion is then centrifugalized until the solids precipitate. The supernatant fluid is siphoned off and discarded. The solid residue is collected, desiccated *in vacuo* over sulphuric acid, mixed with water and after 24 hours centrifugalized as before. The supernatant fluid, second watery extract, is collected and set aside; the residue again desiccated *in vacuo*, after which it is extracted with water for the third time, the same as before and the watery extract set aside.

By repeating in the same way several times more this extraction, and retaining the extracts, eventually there is no residue; the entire bacillary substance having been put in solution.

These several water extracts (solutions) are then mixed.

During the process, the amount of water added each time to residue must be regulated so that, when combined, there will be a total volume 100 cc. of solution for each gram of bacteria with which the production was started.

A measured portion of the combined extracts is dried *in vacuo* and the solid residue weighed to determine the amount of solids per cubic centimeter.

Finally glycerin and water (equal parts) are mixed with the combined watery extracts so that each cubic centimeter of the finished product contains 2 milligrams of solids.

Tuberculin is made from the human tubercle bacillus and from the bovine tubercle bacillus.

In the preparation of all these tuberculins it is a customary precaution to add 0.5 per cent. phenol just before placing in containers for storage or dispensing and when diluting for use 0.5 per cent. phenol solution is the diluent employed.

B. F., B. E. and T. R., tuberculins not subjected to sterilization by heat, are tested for sterility by planting on glycerin agar slants and guinea-pig injections before dispensing.

To determine whether grinding of dried bacteria has been adequate, samples are stained and examined microscopically and grinding continued until such examinations reveal no bacteria that have escaped comminution.

There are many tuberculins which are slight modifications of those introduced by Koch, such as Maragliano's, basically and physiologically the same, possessing slight if any advantages over the Koch products and not generally employed.

Radically different, scientifically of interest to the bacteriologist and immunologist and perhaps worthy of more extensive clinical investigation than has been given it, is Von Ruck's tuberculin.

In the Medical Record, vol. lxxxii, Aug. 31, 1912, Karl Von Ruck describes the preparation, physiological action and dosage of his tuberculin in the following words:

"**Method of Preparing.**—The culture of tubercle bacilli used is of human origin, grown on bouillon, and has been perpetuated in my laboratory for the past 10 years; it was apparently avirulent when first tested upon guinea-pigs and has continued so to the present time. On the manufacture of the preparation heat has been avoided and the chemical effect of light excluded. No chemicals have been introduced in kind or concentration that could injure, split, reduce, or alter the several constituents. The cultures having reached their maximum growth are collected upon a filter and washed free of adhering culture fluid until the filtrate gives no further biurette reaction. The bacillary mass is then transferred to a glass container immersed in distilled water containing 0.4 per cent. phenol, and with frequent stirring and shaking it is macerated for several days, when the filtrate obtained contains the protein designated as No. 1; chemically examined, it shows primary proteose, 25 per cent.; secondary proteose 70 per cent.; peptone, small amount; reaction acid. After further washing with distilled water, the bacilli are dried and powdered, when their fat extraction follows; after drying they are again powdered and then partially extracted in distilled water yielding protein No. 2, this showing coagulable protein, 0.03 per cent. (estimated by nitrogen); primary proteose and deuterproteose in about equal amounts, total about 48 per cent., secondary proteose 50 per cent., peptone trace, phosphorous content 0.1 per cent. and alkaline reaction. The bacillary mass is again dried and powdered and suspended in 0.4 per cent. carbolized distilled water and then ground wet in glass capsules with agate marble, until repeated microscopic examinations no longer show a fragment or formed substance of the bacilli. Filtration through porcelain gives protein No. 3, in solution, differing from No. 2 by absence of coagulable protein, the relatively small amount of primary and increased amount of secondary proteose (75 per cent.)

peptone is shown in small amount. The phosphorous content is 0.5 per cent., the reaction acid.

"Protein No. 4 passes the filter after an addition of an 0.4 per cent. solution of sodic hydrate and gives all reactions for nucleo protein. The remaining bacillary residue amounting to about 5 per cent. weight of fat from tubercle bacilli, is free from proteins (nitrogen determination), may contain traces of fats, and is probably cellulose. The secondary proteoses give reaction for sugar.

"The fatty extractives of tubercle bacilli may be obtained separately as neutral fats and fatty acids by their saponification and subsequent extraction of the acid precipitate with alcohol and with ether, or in order to avoid undesirable chemical changes by saponification, the alcohol fats and the ether fats may be obtained respectively by first complete extraction with ether, followed by alcohol and *vice versa,* which to me seems preferable. When in solution they can be shaken out with distilled water, the later holding about 0.5 per cent. in free suspension, which is opalescent in appearance in that concentration, the opalescence disappears in the concentration employed by me. The several proteins and fatty extractives having been obtained separately and having each been standardized, any free alkali in protein No. 4 having been neutralized by addition of weak solution of HCl, just short of causing precipitation, the several constituents are assembled to represent the formula (given below).

"The precautions employed in preventing injury or undesirable modifications of the several bacillary products have not all been demonstrated as absolutely requisite for their highest efficiency, my purpose in their adoption having been to take no chances of an inferior preparation by their omission.

"**Formula.**—This vaccine is absolutely free from real or supposed danger, powerful enough to act by one or two applications, uniform in action by producing the desired immunity in all cases, simple enough to make it available for use of the general practitioner, without the necessity of elaborate examinations or investigations in selecting suitable cases.

"The vaccine ready for use contains per cubic centimeter 10 milligrams of proteins of tubercle bacilli and a small amount of their fatty extractives, proportioned quantitatively as follows:

Protein No. 1, 0.25 mg.
Protein No. 2, 2.75 mg.
Protein No. 3, 1.00 mg.
Protein No. 4, 6.00 mg.
Fatty extractives, 0.01 mg.

In the adoption of the amounts of the several constituents present in the preparation, I was governed by the study of their action when employed separately in animal experiments as well as in some children. Finding that with the same doses but little effect was noted in the production of specific antibodies in the use of protein No. 1, this is present in the smallest amount, whereas protein No. 2, and especially the nucleoprotein designated as No. 4, appearing most effective, they are quantitatively in excess. The addition of fatty extractives may be eventually found unnecessary, it appearing in my studies of complement fixation with the several constituents of the tubercle bacillus as antigens, that such fixation occurs with all of them inclusive of the fatty extractives with the sera of non-tuberculous children after their vaccination with protein Nos. 2, 3 and 4, irrespective of the presence of one or the other of the fatty extractives in the preparation.

"The dose of vaccine is:

0.05 to 0.10 to nurslings.
0.02 to 0.60 cc. to children. The higher dosage is given to those 18 to 20 years old.

"Usually at point of injection in arm there isn't much swelling or tenderness unless the patient is suffering from an active tuberculous lesion."

In properly selected cases, when properly administered, tuberculin will often establish the nature of a tubercular infection when other methods of diagnosis fail to.

Tuberculin, like other bacterial products, is a potent agent capable of benefiting or seriously injuring those treated with it.

It should only be administered by those familiar with its properties and experienced in immunology.

Koch's old tuberculin is the tuberculin of choice for diagnostic purposes. There are different methods of using it. Its value is based on the fact that, when properly administered, a localized or general reaction occurs in tuberculous patients and no reaction occurs in the non-tuberculous.

For diagnostic purposes tuberculin is injected subcutaneously, rubbed into a scarified cutaneous surface, rubbed into the skin where there is no breach of continuity, or dropped into the eye. Special advantages are claimed for each of these methods and each has its limitations.

The subcutaneous administration of tuberculin gives the most accurate results. It is strictly contraindicated when the patient's temperature is above normal and perhaps should not be used to diagnosticate tuberculosis in children under 10 years of age. Successful employment depends upon accurate observation of the patient's temperature and physical signs before and after the injection of tuberculin.

The degree of reaction manifest by a tuberculous patient following an injection depends upon the sensitiveness of the patient and the size of the dose. This reaction may be nothing more than inflammation at the point of inoculation, some or all of the symptoms and physical signs of disease may be aggravated, there may be a rise of temperature from 0.5°F. to 2°F., or, in a severe reaction, all these manifestations may be observed.

The endeavor of the diagnostician is to obtain a distinct reaction, but as slight a one as possible; a rise of temperature greater than 1°F. is always to be avoided.

Different patients vary to a great degree in sensitiveness to tuberculin, hence the initial dose must be less than could injure the most sensitive, consequently the first, perhaps the second injection, will not cause a reaction in the majority of tuberculous patients.

The technique of the subcutaneous tuberculin test is as follows:

The patient's temperature is taken every 2 hours for 1 or 2 days before injecting and symptoms and physical signs are carefully noted.

The first injection should be 0.0001 cc. or less. Following the injection the patient's temperature should be taken and the physical signs noted every 2 or 3 hours for 24 hours. If no reaction is caused by the first injection a second test is made after an interval of 48 hours, using 0.001 cc. If the second test is negative, subsequent tests are made at intervals of 48 hours, using 0.002 cc., 0.003, 0.004, 0.005, 0.007, 0.009.

Should there be reason to suspect tuberculosis, in spite of negative reactions obtained with tuberculin from the human type, then the tests may be repeated, using tuberculin from the bovine type of the tubercle bacillus.

Von Pirquet Test.—For this test a mixture of Koch's tuberculin 1 part, 5 per cent. aqueous solution phenol 1 part, and normal salt solution 1 part is used.

The patient's arm is cleansed with ether and three areas about 1/8 inch in diameter are scarified. Two drops of tuberculin are placed upon two of these areas and allowed to dry in, care is taken that no tuberculin touches the third, the control area.

The scarified areas must be at least 2 inches apart and the degree of scarification must be exactly the same in each instance. Only the epiderm is scarified, blood must not be drawn. A positive reaction shows redness and edema of the areas inoculated with tuberculin 48 hours after introduction, a papule or vesicle follows and subsides in about a week. The control area shows none of these changes.

The results of the work of the last couple of years suggest that in the near future the intracutaneous tuberculin test as carried out by Craig of London, Canada, will supplant the Von Pirquet. This is substantially as follows:

The patient's arm is cleansed as for a Von Pirquet. A drop of sterile glycerin broth is placed on the skin and driven in by puncturing the epidermis six times with a round, solid, sewing needle. Several inches away a drop of tuberculin is placed on the skin and punctures are made through this. Excess broth and excess tuberculin are wiped away. In making the punctures, care is taken to thrust the needle point into but not through the skin, and blood should not be drawn.

Moro Test.—A 50 per cent. lanolin ointment of old tuberculin is rubbed into the skin of the abdomen over an area of about 6 square centimeters. A positive reaction shows a red papular eruption after 1 or 2 days.

Both the Von Pirquet and Moro test have their greatest value when applied to young children. A large number of apparently healthy adults show positive reactions when subjected to these tests.

Calmette's Ophthalmo Test.—Calmette's ophthalmo test is performed by dropping into one eye 1 minim of a 1 per cent. aqueous solution of purified tuberculin (twice precipitated with alcohol).

This test frequently produces misleading results and is not without danger.

RABIES (Lyssa, Hydrophobia)

Rabies is a disease, primarily of dogs and wolves, which occurs in epizootic form, in cats, cows, horses, swine, sheep, deer and various wild animals.

In man the disease occurs as a result of infection by the bite of a rabid animal—in the vast majority of cases dogs or wolves being the offenders.

The bite of animals afflicted with this disease is infectious from 1 week before they present signs of illness until the termination of the disease.

Not all animals suspected have the disease, and fortunately, only a portion of those bitten by rabid animals are infected.

Rabies is hopeless in man, once clinical signs appear, but it is possible to immunize persons infected, before the termination of the period of incubation, provided prophylactic vaccination is instituted shortly after infection. One can only determine whether or not an animal that has bitten a person has the disease, by microscopic examination of the brain, and subdural inoculation of rabbits with several drops of brain emulsion.

When a suspected animal is killed for such an examination, injury of its head should be avoided. Decapitation should be done so as to leave as much as possible of the neck attached to the head. If the head must be held for some hours or shipped before examination, it should be packed in a bucket of ice..

The entire calvarium and posterior portion of the cervical vertebræ are sawed away and removed without injury of underlying tissue before any attempt is made to remove the brain and medulla.

Films for Microscopic Examination are prepared as follows: An incision is made at right angles to the surface and to the long axis of convolution into the hippocampus major and cerebellum. Pieces of tissue about 1 millimeter thick and several millimeters in diameter are removed, each placed near the end of a glass slide and spread in a thin even film by sliding a second slide over the first one while making gentle pressure. They are immediately immersed in methyl alcohol for fixation. This must be done rapidly to prevent drying and consequent tissue changes. After 3 minutes in alcohol slides are removed and stained for 30 seconds with the following stain, freshly prepared.

Engle's modification of Van Gieson's stain for negri bodies:

Loeffler's alkaline methylene blue................	5 cc.
Distilled water	20 cc.
Saturated alcoholic sol. fuchsin..................	4 drops.

When properly stained the protoplasm of nerve cells is faint blue, nucleus purple and nucleolus dark blue. If the blue is too intense the staining may be corrected by adding more distilled water to the stain and heating slides while staining. Negri bodies occur within the protoplasm of nerve cells, usually one, occasionally several, appear in one nerve cell. They stain maroon red and contain one or more deep purple or black inner bodies.

Negri bodies, first described by A. Negri in 1903, are found in almost 99 percent. of smears from brains of rabid animals, they have never been found in other diseases; their nature is obscure.

Negri bodies are round or oval and vary in size from 1 μ to 20 μ in diameter. The smallest are observed in brain cells of rabbits inoculated with virus fixe; the largest in the brain cells of cows afflicted with rabies. In the brains of dogs dead from street infection they vary from barely visible to about 10 μ.

Inoculation of Rabbits.—As soon as specimens have been removed from a suspected brain for microscopic examination, the brain should be placed in glycerin. If microscopic examination fails to disclose negri bodies, remove brain from glycerin, excise from beneath the surface a piece of cerebellum about one-half the size of a walnut, place in a sterile glass mortar with 15 or 20 cc. of sterile normal salt solution and emulsify by trituration. Allow the suspension to stand for a few minutes so that gross particles precipitate, then draw several cubic centimeters into a glass syringe.

Have an assistant hold a rabbit. With a sharp scalpel make an incision about ½ inch long through the skin in the median line midway between the eyes. Bore a small hole through the bone and thrust the tip of the needle through the dura and inject several drops with the syringe. Seal the incision with collodion and place rabbit in a closed observation cage. The period of incubation is usually less than 3 weeks, occasionally it is prolonged.

The onset of the disease may be marked by maniacal excitement followed in 24 to 72 hours by progressive paralysis and death. The stage of excitement may not occur, the first evidence of the disease being paralysis of the hind quarters and muscles of deglutition—"dumb rabies"—the paralysis progressing and terminating fatally in 3 to 5 days.

The specific cause of rabies is still a mooted point. Some believe the negri bodies the specific cause (not likely). F. Proescher and others have isolated from the brain and cord of rabid animals a small coccus which may or may not be the cause. Probably not.

The causative virus, whatever its nature, is present in the saliva and in the spinal cord and brain of infected animals several days before the onset of the disease. It resists antiformin and glycerin, and can be transmitted from animal to animal.

Pasteur discovered that after passing the virus from one rabbit to another through a series of about 40 or 50 rabbits, the virus finally acquires certain new, permanent properties and is then known as "Virus Fixe."

Rabies vaccine is made from the spinal cord or brain, removed from a rabbit dead from the effect of inoculation with virus fixe.

The amount of virus in such brain or cord is attenuated by suspending it in a glass jar over a layer of potassium hydrate at 22°C. The longer it is so dried the greater the attenuation, until the virus entirely disappears, which it will do in from 3 to 4 weeks, according to the thickness of the cord.

Pasteur's method of administering rabies vaccine to man is about as follows:

Day of treatment	Cord that has been dried, days	Amount
1st day, 10 A M.	14 or 13	3
1st day, 4 P.M.	12 or 11	3
2d day, 10 A.M.	10 or 6	3
2d day, 4 P.M.	8 or 7	3
3d day, 10 A.M.	6	2
3d day, 4 P.M.	6	2
4th day, 10 A.M.	5	2
5th day, 10 A.M.	5	2
6th day, 10 A.M.	4	2
7th day, 10 A.M.	3	1
8th day, 10 A.M.	4	2
9th day, 10 A.M.	3	1
10th day, 10 A.M.	5	2
11th day, 10 A.M.	5	2
12th day, 10 A.M.	4	2

Day of treatment	Cord that has been dried, days	Amount
13th day, 10 A.M.	4	2
14th day, 10 A.M.	3	2
15th day	5	2
16th day	5	2
17th day	4	2
18th day	4	2
19th day	3	2
20th day	3	2
21st day	3	1

When the above method of treatment is to be carried out, a cord which has been dried for the designated number of days is removed from the jar, about a third of it cut off and ground up in a mortar with salt solution until emulsified; the emulsion is allowed to stand at rest for a number of minutes until gross particles precipitate, then the amount of the supernatant fluid to be injected is drawn into a hypodermic syringe and injected into the abdominal wall. In recent years it has been shown that an emulsion of undried brain or cord, containing many times the total amount of virus injected in 3 weeks by the Pasteur method, may be given as a first injection without producing any untoward effects, and without danger of producing rabies. At the present time, in some hospitals, treatment is completed in 5 days by giving daily injections of an emulsion of undried brain or cord.

Rabies vaccine is employed to immunize against rabies. The treatment should be instituted at the earliest possible moment after a probable infection.

If rabies vaccine is not given in time to produce immunity before the onset of symptoms of the disease it is useless.

SMALL-POX VACCINE

Small-pox vaccine is the serum obtained from a vesicle on the skin of a calf suffering with cow pox.

This serum is employed to produce immunity against small pox. The arm of the person to be inoculated is washed with soap and water and alcohol, dried and scarified without drawing blood. Several drops of the serum are rubbed into the scarified area and allowed to dry there.

When the desired effect occurs, redness and swelling develops at the point of inoculation in about 3 days; several days later a vesicle appears; this is transformed into a pustule in the course of several days, and the pustule gradually dries, a dark scab forms and healing occurs so that the entire process disappears in from 8 to 16 days.

COLEY'S FLUID

Coley's fluid is used in the treatment of inoperable cases of sarcoma. It is injected subcutaneously at the margin of the tumor or at any other point. The initial dose is ¼ minim; in the course of several hours a marked reaction of short duration follows.

An injection is given daily, the dose being increased as rapidly as possible without producing reactions.

Grow bacillus prodigiosus on agar at room temperature for 10 days. Scrape off the growth with a glass rod and rub to a paste in a glass mortar, using normal salt solution as diluent, bottle and sterilize at 75°C. for 1 hour. Place in ice box. Grow virulent streptococci in bouillon at 37°C. for 10 days.

Determine the amount of nitrogen per cubic centimeter in the prodigiosus paste to ascertain the amount of protein per cubic centimeter and so dilute it with normal salt solution that each cubic centimeter of the vaccine will contain 2.5 mg. of protein.

To each 100 cc. of the bouillon culture of streptococci add 30 cc. of prodigiosus suspension and 20 cc. of glycerin. Place in bottles, add a crystal of thymol to each and sterilize in a water bath at 75°C. for 2 hours.

HAFFKINE'S VACCINE

Haffkine's vaccine is made by planting the bacillus pestis in plain bouillon to which sufficient sterile oil or butter has been added to cover the surface with numerous droplets. The culture is incubated at 35°C. to 37°C. where there is little or no vibration. Growth occurs around and adheres to the droplets of oil, forming white stalactites that hang from the surface as icicles hang from the roof of a tunnel. When a crop of stalactites forms, the flask is shaken and they fall to the bottom.

The flask is again incubated and a second crop of stalactites forms and is shaken down.

When the fifth or sixth crop of stalactites has formed the flask is shaken until they are broken up and the bacteria evenly distributed throughout the medium. The flask is then submerged in a water bath and heated at 75°C. for 2 hours. The vaccine is then ready for use.

Haffkine's vaccine is administered to healthy people to immunize them against plague. It confers almost complete immunity to the bubonic but only slight immunity to the pneumonic form of plague.

Two subcutaneous injections are given, the first 2 cc. to 3 cc. and 10 to 15 days later from 3 cc. to 5 cc.

ANTHRAX VACCINE

Plain broth cultures are incubated at 42°C. until they become avirulent for rabbits and guinea-pigs but are still lethal for mice. From cultures so attenuated, vaccine No. 1 is made, it being a subculture in broth inoculated at 37.5°C.

Other cultures are attenuated by incubation at 42°C. until avirulent for rabbits but still lethal for both guinea-pigs and mice. From cultures of this attenuation vaccine No. 2 is made, it being 48-hour-old subculture in broth incubated at 37.5°C.

Cattle and sheep are successfully immunized to infection with the anthrax bacillus by two subcutaneous injections a fortnight apart. Vaccine No. 1 is employed for the first injection and vaccine No. 2 for the second. It is not employed *in homo.*

CHAPTER VII

THERAPEUTIC SERA

Diphtheria Antitoxin

When a meat-infusion bouillon culture of the diphtheria bacillus, which has been incubated at 37°C. for several weeks, is filtered through a porcelain filter, the filtrate contains the toxin, but no bacteria, and we speak of this filtrate as toxin. It is capable of producing disease and death when injected into susceptible animals in sufficient quantity.

Different cultures of diphtheria bacillus produce different quantities of toxin, consequently when toxin is obtained by filtering a bouillon culture, its strength must be determined. A unit of diphtheria toxin is the smallest amount that will regularly kill a 250-Gm. guinea-pig in 4 days; this amount is called the "Minimum Lethal Dose," expressed "M.L.D."

The first step in the production of diphtheria antitoxin is to produce toxin and determine its M.L.D. The next is to immunize a horse. This is done by injecting a minute quantity of the toxin into a horse. About a week after the first injection the horse is less susceptible to the toxin than before and a second injection, larger than the first in amount, is given. At intervals of about a week subsequent injections of gradually increasing amounts of toxin are given until, in 2 or 3 months, the horse acquires maximum immunity. Then, under aseptic conditions, the horse is bled and the serum obtained from the blood. The antitoxic properties of the serum are confined to the serum pseudoglobulins, the pseudoglobulins are removed from the serum by mixing the serum with an equal amount of saturated ammonium sulphate solution; this is allowed to stand for 12 hours and the precipitate is then collected on hard filter-paper.

The precipitate is dissolved in sufficient water to bring it up to the original volume of the serum, an equal amount of saturated ammonium sulphate solution is added and, after 12 hours, the precipitate is collected and dried between filter-papers until it has a pasty consistency. It is then dissolved in a saturated solution of sodium chloride. This solution is filtered and 2.5 cc. of 80 per cent. acetic acid is added to each liter of the filtrate. The precipitate is collected and dried on filter-papers and dialyzed in running water until dissolved. It is then neutralized and again dialyzed until free of sodium chloride.

The amount of antitoxin present in the serum of immunized horses varies, hence when serum is obtained its strength must be determined. This is done by mixing various amounts of serum with a M.L.D. of toxin and injecting the mixtures into guinea-pigs, until an amount is found which is just sufficient to neutralize a M.L.D. of toxin.

A "unit" of diphtheria antitoxin is 200 times the amount necessary to neutralize one M.L.D. of toxin.

Diphtheria antitoxin is employed as a prophylactic and as a curative agent.

When given as a prophylactic the immunity to infection conferred is a passive immunity and hence lasts only a few weeks.

Diphtheria antitoxin is specific for the treatment of diphtheria. The earlier it is given in the disease the better the results.

When given as a prophylactic and in mild, early cases of the disease it is injected subcutaneously, preferably in the left side of the abdominal wall. In late, severe cases it is injected into a vein.

The prophylactic dose is from 1000 to 2000 units, the curative dose from 10,000 to 40,000 units; repeated, if necessary.

TETANUS ANTITOXIN

Tetanus antitoxin is produced in practically the same way as diphtheria antitoxin. Selected horses are injected at intervals with increasing amounts of tetanus toxin (filtrate from bouillon culture of tetanus bacillus) until maximum immunity is acquired. The horse is then bled and the blood serum collected.

The American unit of tetanus antitoxin is 10 times the amount necessary to immunize a 350-Gm. guinea-pig against 100 times the M.L.D. of toxin.

Tetanus antitoxin has its greatest value when employed as a prophylactic, injected subcutaneously near the wound as soon as possible after the occurrence of a wound or trauma likely to be infected with the tetanus bacillus, such as black powder burns, deep puncture wounds inflicted by splinters of wood from old buildings, rusty nails or instruments contaminated with garden soil, stable dirt or hide dust.

The prophylactic dose is from 1000 to 2000 units; this may be repeated in a week if deemed necessary.

When symptoms of tetanus have developed, antitoxin seldom prevents a fatal result; it must be used in heroic doses, preferably injected into the spinal canal.

ANTISTREPTOCOCCUS SERUM

Antistreptococcus serum is produced by injecting horses with killed and attenuated streptococci until maximum immunity is acquired. As many different strains of streptococci as can be procured are used, to produce a polyvalent serum.

The immunizing power of such serum is said to depend upon the opsonin content.

No definite dosage has been established. Large quantities are required to obtain therapeutic effect, an initial dose of 100 to 200 cc., followed by repeated doses of 10 to 20 cc. every 4 hours, if the first dose proves beneficial.

Antistreptococcus serum should be injected deep into the muscles.

The indications for its employment are said to be acute, severe primary and secondary streptococcus infection.

Antistreptococcus serum does not have the value of some other antitoxic and bacteriocidal sera. Sometimes it produces excellent results and at other times it exerts no influence on the course of a streptococcus infection.

ANTIMENINGOCOCCUS SERUM

Antimeningococcus serum is produced by injecting horses, alternately, with killed meningococci and solutions prepared as follows:

Pour 10 to 20 cc. of normal salt solution on a 24-hour-old culture of meningococci, shake until the bacteria are suspended in the salt solution, place this salt solution suspension in the incubator at 37°C. for 1 day and then inject. After the horse has been injected at intervals for a couple of months with killed meningococci, he is then injected with living meningococci for about 6 months.

Antimeningococcus serum is the specific for the treatment of spinal meningitis caused by the meningococcus. It is injected into the spinal canal, the quantity being determined and only limited by the patient's tolerance, from 10 to 50 cc.

ANTIANTHRAX SERUM

(SCLAVO'S SERUM)

Sclavo's serum is produced by injecting sheep or horses first with sensitized bacilli of attenuated virulence, then with attenuated bacilli, not sensitized, finally with virulent bacilli.

The serum of animals so immunized is of great value in the treatment of anthrax. It should be employed as early as possible. The amount used is large, from 20 to 100 cc. repeated if necessary. A marked reaction follows the first injection of Sclavo's serum. In grave cases and those coming under treatment late in the disease, the serum may be injected into a vein; in less urgent cases intramuscular or subcutaneous administration is practiced.

When injected subcutaneously it takes about 10 months to immunize a horse so that it may be bled. Amos and Wollstein report a quicker method. By giving intravenous injections of the autolized products of meningococci and para-meningococci, and living virulent cultures of these organisms, they have produced therapeutic serum in 3 months.

CHAPTER VIII

WASSERMANN AND OTHER COMPLEMENT FIXATION TESTS

APPARATUS

The apparatus needed for complement fixation tests:

An electric centrifuge, a hot-air sterilizer, a steam sterilizer or autoclave, a balance sensitive to 0.1 Gm., an incubator, a small water bath, one dozen graduated centrifuge tubes (15 cc. capacity), half a dozen wide-mouthed ground-glass-stoppered bottles of 500 cc. capacity, a like number of similar bottles of 200 cc. capacity, 50 florence flasks (100 cc. capacity), 50 deep petri dishes (4 inches in diameter), 100 glass vials (2 cc. capacity), and rubber stoppers for same, 100 test-tubes of each of the following sizes: 6 by 1 inch, 6 by 3/4 inch, 4 by 3/8 inch, several standardized cylindrical graduates (1000 cc. capacity) graduated in 10 cc., a half dozen pipettes (5 cc. capacity) graduated in 1/20 cc., a dozen pipettes (1 cc. capacity) graduated in 1/100 cc. (these pipettes should discharge between graduation marks and should not be graduated to the tip), several graduated Luer glass syringes (2 cc. capacity), several 10-cc. capacity Luer glass syringes, 100 Gm. chemically pure sodium chloride, 100 Gm. chemically pure sodium citrate, chloroform, ether and non-absorbent cotton.

Needles are also required. Either steel or iridium-platinum needles may be used. The initial cost of the former is very much less than the latter, but eventually the less corrosive are probably the cheaper. The iridium-platinum needles are also the nicer to work with. For the 2-cc. syringes, needles about 1 1/2 inches long, with the smallest bore through which blood cells can be passed, are required. The needles for the 10-cc. syringes are used for withdrawing blood from the veins of patients and from the hearts of rabbits or guinea-pigs. It is best to have several sizes of these, the most useful being the larger-size serum needle.

All glassware used in this work should be thoroughly washed in several changes of distilled water and be free of acids or alkalies; it should be set aside for this work exclusively and thoroughly washed in plain water immediately after use. For some complement fixation tests it is essential that apparatus be sterile. It is a good practice to follow in all these tests even when not absolutely necessary, and it is, therefore, recommended that all containers—flasks, bottles, test-tubes, pipettes, syringes and needles be sterilized immediately after use and kept in sterile containers.

In our own laboratory all empty glassware is sterilized in a hot-air sterilizer at 140°C. for 2 hours. Before sterilization, pipettes are placed in copper containers or wrapped in paper; Petri dishes and syringes are enveloped in double paper wrappers, tubes used for fixation tests are wrapped in paper in packages of six, all other tubes are plugged with cotton, flasks are plugged with cotton, and flasks and bottles are also capped. Normal salt and sodium citrate solutions are autoclaved at 15 pounds pressure for 20 minutes or heated 20 minutes each day for 3 consecutive days in a steam sterilizer. Needles are kept in 50 per cent. alcohol. They are flamed just before use.

The form of test-tube rack is a matter of personal selection; the author prefers a heavy, rigid, metal rack built to carry three tiers of 12 tubes each, in alignment (Fig. 36).

INCUBATOR

Biochemical reactions, such as complement fixation, proceed best at temperatures that approximate normal body temperature—37°C.—consequently an incubator that will constantly maintain this temperature is necessary.

NORMAL SALT AND SODIUM CITRATE SOLUTIONS

When red cells (corpuscles) of the blood are removed from their natural vehicle (blood serum) and placed in another fluid, their normal properties are retained, altered or destroyed according as to whether the fluid in which they are placed is isotonic, hypertonic or hypotonic.

If red cells are placed in water they immediately burst, liberate their hemoglobin and dissolve; if they are placed in **normal salt solution** they can remain viable and retain all their normal properties for days. Red cells, separated from their native serum, are required in the Wassermann and other Complement Fixation tests; hence **normal salt solution** is the sole vehicle and dilutent used in this work.

Normal salt solution is made by dissolving 8.5 Gm. of sodium chloride in 1000 cc. of distilled water. To preclude undesirable changes **normal salt solution** should be sterilized as soon as it is made and stored in sterile containers having a capacity of not more than 200 cc.

If blood is withdrawn from an animal and placed in a container it will clot in a few minutes; the corpuscles are embedded in the clot and cannot be extricated. Since red blood cells are essential in making complement fixation tests, it is necessary to collect the blood in a manner that will prevent coagulation. **Sodium citrate** precipitates the calcium salts of the blood and so prevents its coagulation; hence **sodium citrate solution** is used in collecting blood from which to obtain red cells for complement fixation tests.

Sodium citrate solution is made by dissolving 10 Gm. of sodium citrate in 1000 cc. of **normal salt solution.** **Sodium citrate solution** should be sterilized when made and stored in sterile containers.

BLOOD CELLS AND SENSITIZATION OF RABBITS

After procuring the necessary apparatus and preparing normal salt and sodium citrate solutions, the next step in preparing for complement fixation tests is the sensitization of a rabbit against red blood cells. The rabbit must be sensitized to the same kind of red blood cells that will subsequently be used in performing the complement fixation tests. Sheep's red blood cells and human red blood cells are those most commonly used. So far as the value and end results of complement fixation tests are concerned it makes no difference whether human or sheep cells are used, statements to the contrary notwithstanding. If one is situated where sheep's cells can be procured they should be used, under other circumstances human cells should be used. If one has access to a sheep slaughter house it is most convenient and economical to obtain the blood there. Sheep kept in the laboratory for this purpose may be bled every week or 10 days without injury. This is done by thrusting a strong, heavy, sharp, hollow needle into the jugular vein after the neck has been shaved. After the needle is withdrawn no dressing other than styptic collodion need be applied to the wound. When blood is withdrawn from a man for the purpose of obtaining cells, the most prominent superficial vein is sought for, usually at the elbow, the skin overlying it is washed with alcohol, tincture of iodine applied for 1 minute and the area again washed with alcohol, a sterile hollow needle is then thrust into the vein and the blood allowed to flow from the needle.

All the red blood cells required at any one time will be contained in 50 cc. of blood. The blood is collected as follows: A clean sterile glass bottle (200 cc. capacity) having a wide mouth and a ground-glass stopper is *half filled* with **sodium citrate solution**(Fig. 37A); blood is allowed to flow into the bottle until it is three-fourths full (Fig. 37C)—it must never be allowed to overflow—the stopper is inserted and the bottle shaken to thoroughly mix its contents; it is at once transported to the laboratory and kept in a refrigerator until used. The length of time blood so collected remains fit for use varies from 1 day to several weeks, hence it is always advisable to obtain it fresh each time it is required.

To separate the red blood cells the citrated blood is poured into 15-cc. capacity tubes (Fig. 38A) and centrifugalized until all the cells settle; when this has taken place the lower portion of the tubes will show a solid red opaque mass (the cells) above which will be clear, colorless, transparent fluid (serum and citrate solution) (Fig. 38B). The supernatant fluid is withdrawn with a pipette, care being taken not to disturb the cells (Fig. 38C, D). The remaining cells still have some serum and citrate solution surrounding them, this must be removed; it is done by washing. The cells are washed as follows: The tubes containing the cells are filled to within 1 or 2 centimeters of the top with **normal salt solution,** a clean thumb is placed across the top of the tube to confine its

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EXPLANATORY REMARKS TO FIG. 36.

A1, A2 and A3. These tubes are for one patient's serum under examination.

B1, B2 and B3. These tubes are for a second patient's serum under examination.

D1, D2 and D3. These tubes contain the known syphilitic patient's serum and are, therefore, control tubes.

E1, E2 and E3. These tubes contain non-syphilitic patient's serum and are, therefore, control tubes.

A1, B1, D1 and E1 contain no syphilitic antigen, therefore these tubes should show an illustrated complete hemolysis.

E2 and E3 show complete hemolysis, indicative of a negative reaction.

D2 shows no hemolysis.

D3 only partial hemolysis indicative of a weak positive reaction.

B2 and B3 show no hemolysis. A strong positive reaction indicative of syphilis.

A2 and A3 show complete hemolysis, a negative reaction not indicative of syphilis.

J1 is a complement control tube. Contains nothing but guinea-pig serum and red cells. This tube should show no hemolysis as indicated.

J2. Hemolytic system control, complete hemolysis.

J3. Syphilitic antigen control. Complete hemolysis (see pages 243 and 247).

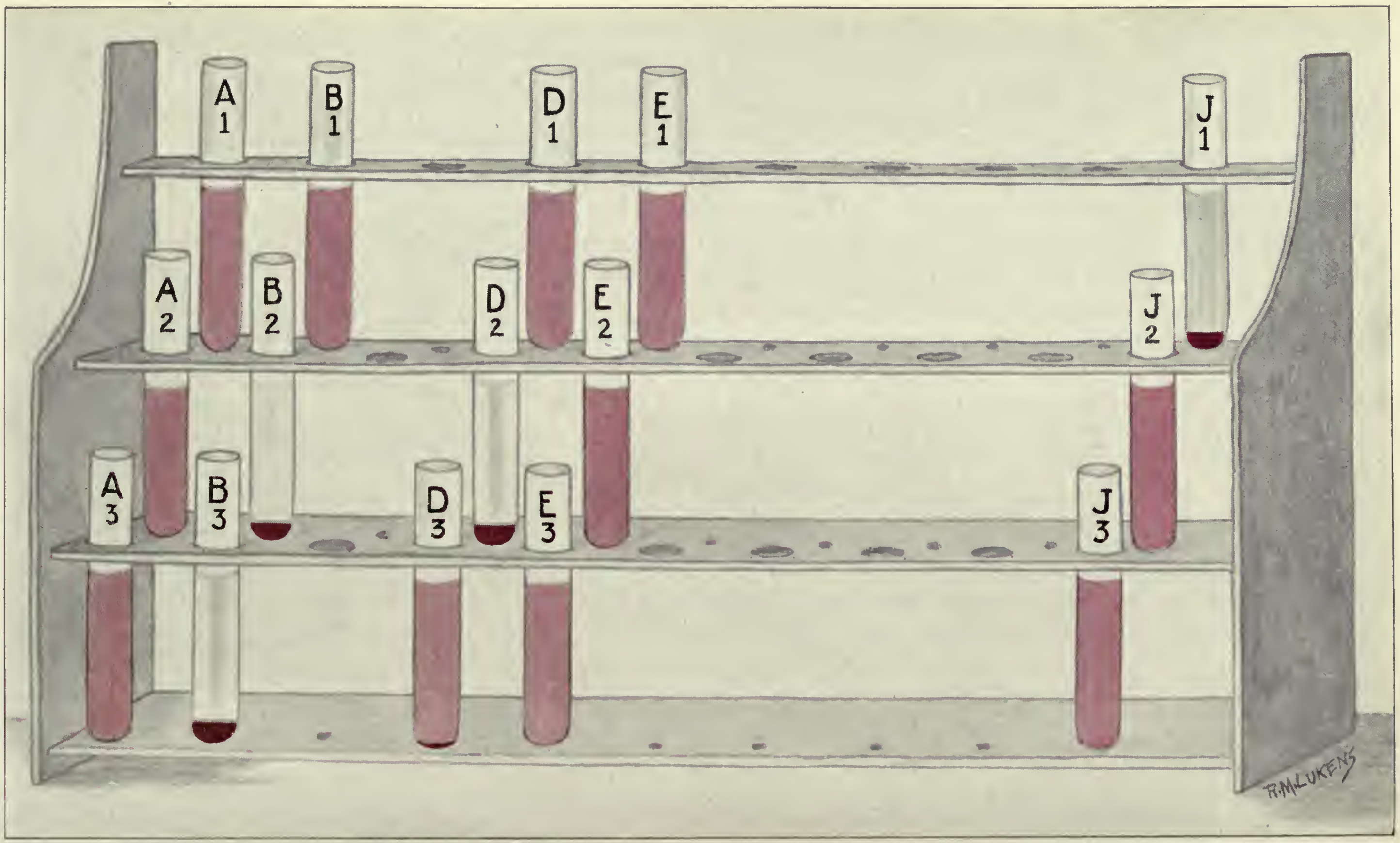
A 1
B 1
D 1
E 1
J 1
A 2
B 2
D 2
E 2
J 2
A 3
B 3
D 3
E 3
J 3
R.M.LUKENS

FIG. 36.—COMPLETED WASSERMANN TEST.

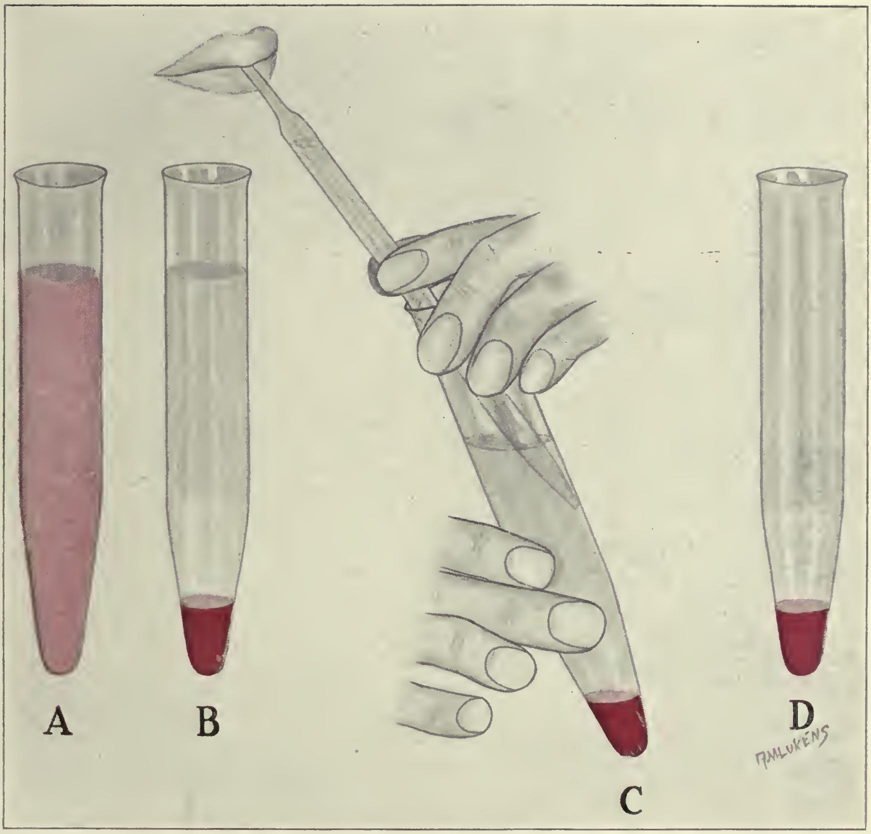

Fig. 38.

A, Citrated blood as poured into centrifuge tube. B, Cells precipitated to the bottom of the tube, clear serum and citrate solution above. Result of centrifugalizing A. C, Removal of supernatant fluid. D, Test tube containing red cells after supernatant fluid has been removed.

contents and the tube is then inverted and shaken a sufficient number of times to evenly distribute the cells throughout the fluid; the tubes are again centrifugalized until all the cells settle to the bottom; the salt solution is pipetted off. This washing is repeated twice. After the third washing the cells are ready to be injected into the rabbit.

Only large healthy rabbits should be used. With a pair of scissors the hair is removed so as to expose one of the veins on the margin of the ear. Half a cubic centimeter of red blood cells is poured into a small (2 cc. capacity) Luer syringe, and with the finest needle through which the cells will pass, they are injected into the vein (Fig. 39).

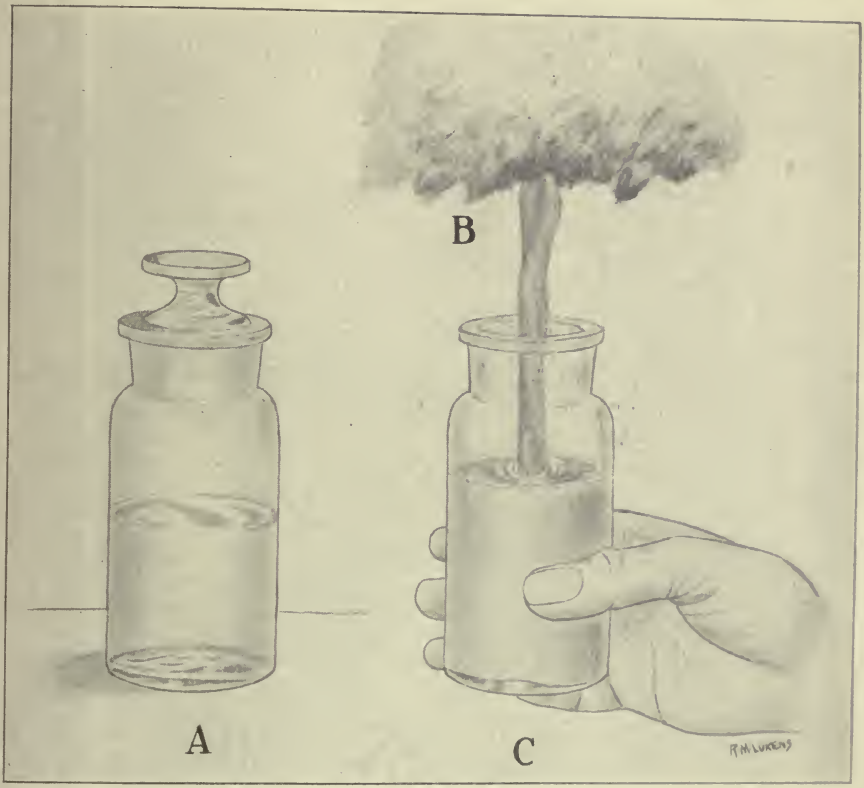

FIG. 37.

A, Sterilized glass bottle with wide mouth and ground glass stopper, half filled with sodium citrate solution. B, Blood from sheep flowing into bottle (C) until the bottle is three-fourths full.

Twenty-four hours after the first injection, in exactly the same manner, the second is given, the quantity of red blood cells injected being 1 cc.

Twenty-four hours after the second injection a third is given—quantity 2 cc.

Twenty-four hours after the third injection the last one is given—quantity 3 cc.

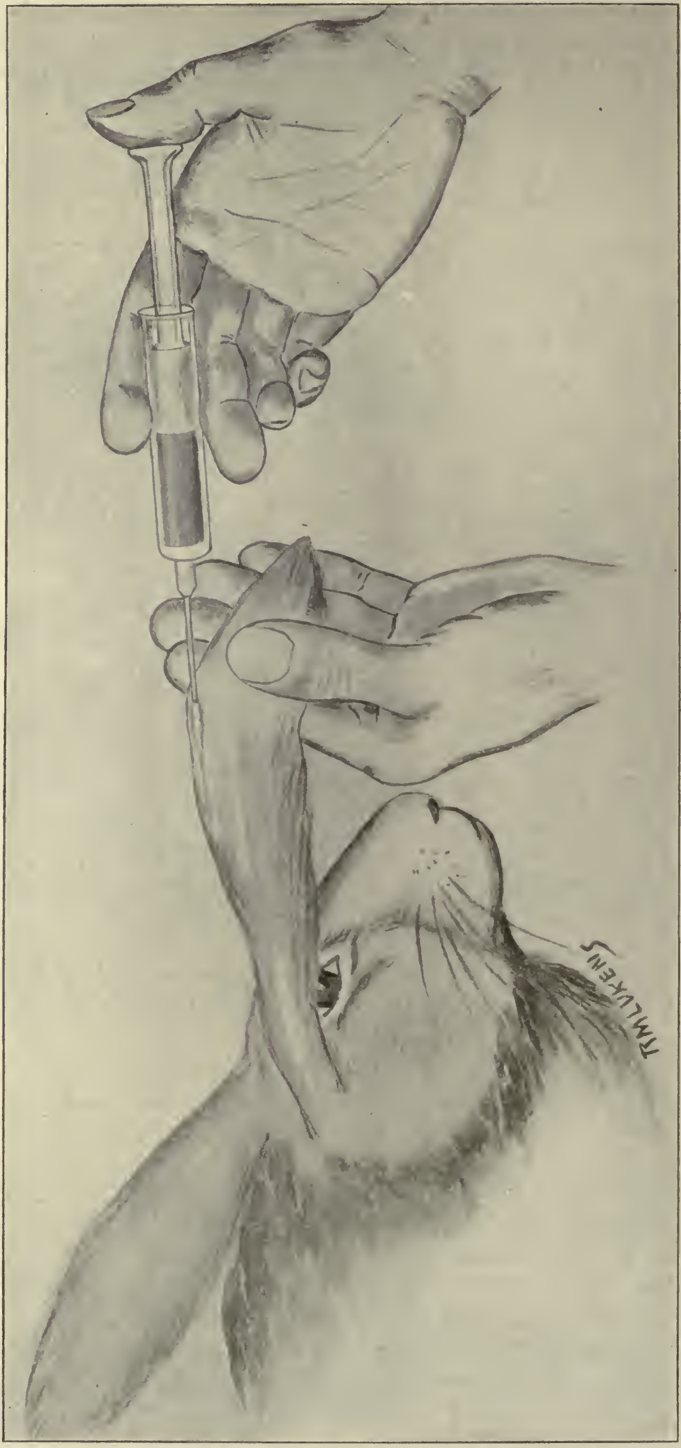

FIG. 39.—INJECTING WASHED RED CELLS INTO VEIN AT MARGIN OF RABBIT'S EAR.

The rabbit is then allowed to rest for 10 days, during which time he develops specific antibodies for red blood cells, such as were injected. When sensitized the rabbit is anesthetized with ether and bled by one of the following methods:

First.—By touch locate the heart, thrust a sterile needle into it and with a 10-cc. Luer syringe withdraw 30 cc. of blood (Fig. 40). Transfer the blood from the syringe into a sterile Petri dish. Inject 30 cc. of warm normal salt solution into the rabbit's peritoneal cavity (Fig. 41).

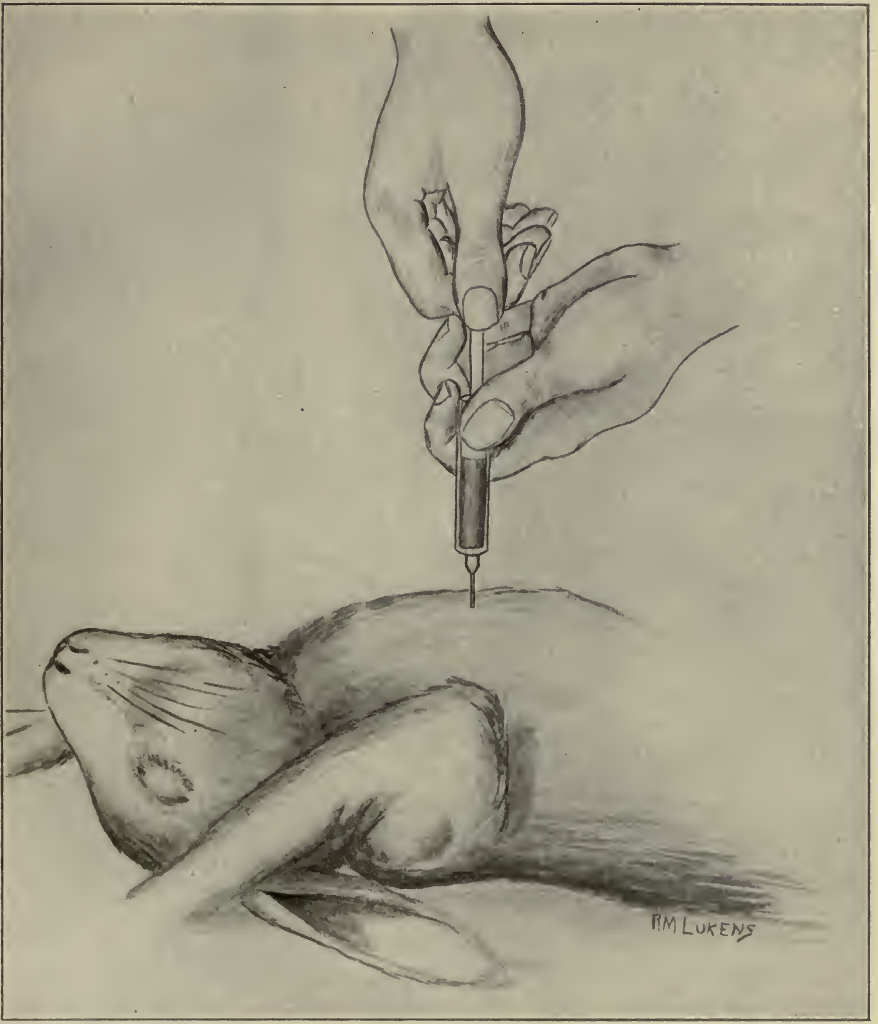

FIG. 40.—WITHDRAWING BLOOD FROM THE HEART OF AN ANESTHETIZED RABBIT.

Second.—Incise the skin at the tip of the sternum with a pair of scissors, one blade of which has a blunt end and the other a pointed end. Elevate the skin and thrust the blunt end of the scissors into the incision, pushing it in the median

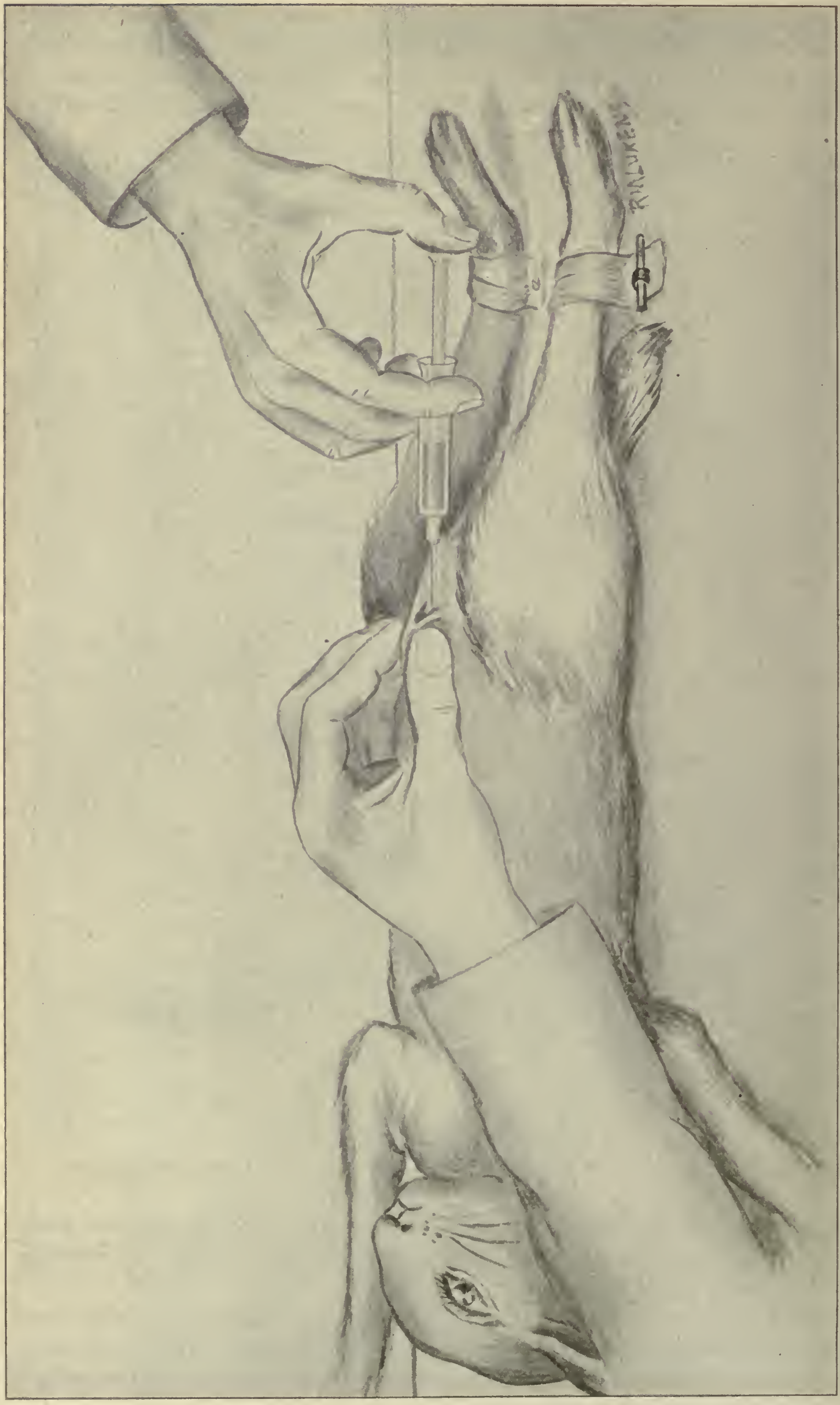

FIG. 41.—INJECTING NORMAL SALT SOLUTION INTO THE PERITONEAL CAVITY OF RABBIT AFTER THE WITHDRAWAL OF BLOOD.

line toward the rabbit's head. The scissors must be kept between the skin and superficial fascia. When the skin has been divided from the sternum to the chin, it is rapidly dissected back until the anterior half of the rabbit's neck is denuded. The rabbit is then grasped by the hind legs, held vertically over a Petri dish so that the blood will flow directly into it when the jugular veins are cut. Care must be taken to avoid opening the trachea or esophagus when incising the jugular veins (Fig. 42).

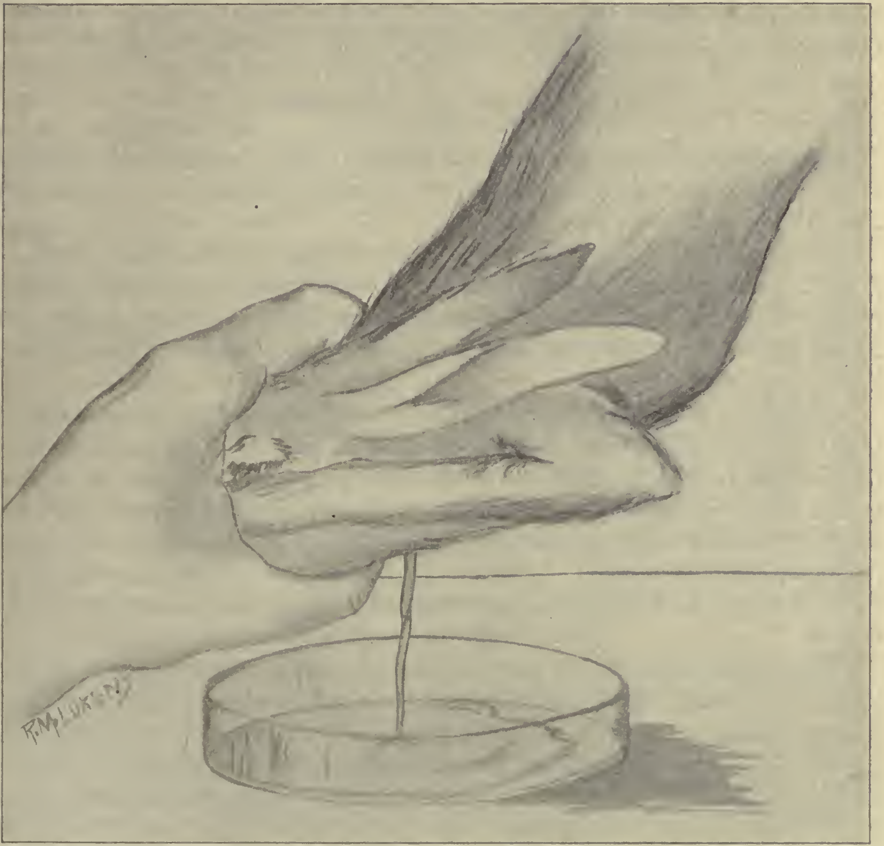

FIG. 42.—BLOOD FLOWING FROM INCISED JUGULAR VEIN OF RABBIT INTO PETRI DISH.

The Petri dishes containing the blood are covered and placed in a refrigerator for from 12 to 24 hours. At the expiration of this time the Petri dishes are removed from the refrigerator and the serum (fluid) is transferred to tubes and centrifugalized until perfectly clear and free of suspended matter. It is then transferred to clean tubes which are placed in a water bath at a temperature of 55°C. or 56°C. for ½ hour. When removed from the water bath about 1 per cent. of chloroform is added to the serum and it is then put in 1-cc. capacity vials which are tightly stoppered with rubber plugs, then stored in a refrigerator.

DILUTION OF BLOOD CELLS

In performing complement fixation tests, as hereafter described, red blood cells are required. They are procured as described in the preceding chapter and are washed twice. After the second washing a 5 per cent. suspension is made by mixing each cubic centimeter of red blood cells with 19 cc. of normal salt solution. When red blood cells are to be added to a tube, invariably, 1 cc. of a 5 per cent. suspension is the amount added. To avoid cumbersome expression, the term **"red cells"** will hereafter be used in this volume to designate 1 cc. of a 5 per cent. suspension of red blood cells in normal salt solution.

COMPLEMENT AND AMBOCEPTOR

Experiment No. 1.—Take a normal healthy rabbit that has been subjected to no treatment, obtain some blood from it, place this blood in a refrigerator until it clots and serum separates from it.

Into a number of test-tubes put various amounts of this serum—from 0.1 to 2.0 cc., add **red cells** to each tube. Shake the tubes to mix their contents. Note their appearance. Place the tubes in an incubator for 1 or 2 hours, then inspect them and transfer them to a refrigerator; several hours later make a final inspection.

Before they were placed in the incubator these tubes showed a homogeneous, bright red, opaque fluid and no precipitate. At the end of incubation the extreme upper zone of the fluid was clear, colorless and transparent, on the bottom of the tubes there was a slight, dark red precipitate and between these two the fluid was still bright red and opaque. At the final inspection a solid, dark red, opaque sediment, about ⅛ inch in depth, covered the bottom of the tubes; above this the fluid was clear, colorless and transparent—like water; as B2, Fig. 36.

When the serum and cells were mixed in the tubes by shaking, the cells were evenly distributed throughout the fluid, hence the homogeneous, bright red, opaque appearance and absence of sediment. When these tubes had stood at rest for an hour or two in the incubator the cells gradually precipitated; the precipitation being only partial, but a slight zone at the top of the fluid was entirely free of cells, hence only this slight zone was clear, colorless, and transparent like water, and only a slight sediment was visible at the bottom of the tubes.

When the tubes were finally inspected, after standing at rest in the incubator and ice box for from 4 to 6 hours, sufficient time had elapsed for precipitation to be complete, in which case all the cells were on the bottom of the tube; all the fluid being free of cells consequently had the appearance of water.

The above experiment furnishes visible evidence that fresh, unheated serum from a normal untreated rabbit has no effect on red blood cells—whether they be obtained from a man, sheep or ox.* This is referred to as **no hemolytic** effect or **no hemolysis.**

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* Rabbit serum may occasionally contain natural Hemolysin for foreign red cells.

Experiment No. 2.—Sensitize a rabbit to red blood cells as described in the preceding chapter; obtain some of its blood, allow this blood to stand in a refrigerator until serum separates; collect the serum and put various amounts of it in test-tubes—from 0.001 to 1.0 cc., add **red cells** to each tube; shake the tubes to mix their contents; inspect them, their appearance will be identical to that observed prior to incubation in **experiment** No. 1.

Place the tubes in an incubator for 1 to 2 hours, then remove and again inspect; there will be a radical change in their appearance. Some of the tubes containing the larger amounts of serum will show a clear, transparent, bright red solution, the same in appearance throughout and there will be no sediment whatever in these tubes. Some of the tubes containing the smallest amounts of serum will present the same appearance as was observed at the end of incubation in **experiment** No. 1. Between these two groups of tubes will be a number in which, as the amount of serum increases, the amount of opaque fluid diminishes, the amount of sediment diminishes and the amount of clear transparent red fluid increases.

When tubes to which red blood cells have been added show a clear, transparent red fluid and no sediment, no matter how long the tubes stand at rest, it is evidence that the red blood cells have been disintegrated and their hemoglobin gone into solution; this change is termed **hemolysis,** and the tubes have the same appearance as A1, Fig. 36.

Experiment No. 2 shows that injection of red blood cells, from another species, into rabbits, stimulates the production of **antibodies**; that these **antibodies** are present in the blood serum of such rabbits; that when this serum in the fresh state and proper amount is added to red cells such as were used to stimulate the production of **antibodies,** it will destroy or **hemolize** the red cells.

Experiment No. 3.—Repeat Experiment No. 2, all factors being the same except that the rabbit serum be heated in a water bath at 55°C. or 56°C. for ½ hour immediately before mixing with the red cells.

There will be **no hemolysis.** This demonstrates that heating the serum containing **hemolytic antibodies** so alters the **antibodies** as to deprive them of their **hemolytic** power.

Experiment No. 4.—Repeat Experiment No. 3, all factors being the same, except that in addition to placing various amounts of heated-sensitized-rabbit serum and red cells in every tube, 0.1 cc. of fresh unheated serum, derived from an untreated, unsensitized normal rabbit, is added to each tube before incubation.

When these tubes are examined after incubation **hemolysis** will be observed the same as in Experiment No. 2—evidence that the **antibodies,** present in the serum of rabbits sensitized to red blood cells, are composed of two parts—one which is destroyed by heating to 55°C. for ½ hour, called **complement**; the other which is not injured, altered or destroyed by so heating, called **amboceptor.**

The experiment also shows that **complement** is a native constituent of all rabbits, untreated and unsensitized as well as those that have been treated or sensitized; and that the complement in one rabbit's serum can unite with the **amboceptors** of another rabbit's serum.

By varying and extending these experiments it can be demonstrated that **complement** is present in the blood serum of all warm-blooded animals throughout life. The amount of **complement** present per cubic centimeter in the blood serum is about the same in any given animal at all times. The amount of **complement** per cubic centimeter in the serum of one animal is nearly the same in all other animals of the same species, but variations do occur. The **complement** present in the serum of an animal can unite with any **amboceptor** present in the serum of that animal or any other animal of the same species. **Complement** of identical nature is possessed by several species and is therefore capable of uniting with **amboceptors** found in any of them. The **complement** present in the serum of guinea-pigs, rabbits and man is practically the same, therefore, guinea-pig **complement** can unite with **amboceptors** present in human serum, and those in rabbit serum. **Complement** is at once destroyed by heating serum to 55°C. for ½ hour; it gradually leaves the serum after the serum is withdrawn from an animal, even though the serum is not heated. If serum is allowed to stand at room temperature it loses its **complement** in about 24 hours or less. If serum is placed in a refrigerator immediately after taken from an animal it retains its **complement** for from 24 to 72 hours. If serum is frozen immediately after taken from an animal it retains its **complement** for a number of days.

The **complement-amboceptor** experiments demonstrate that a given amount of **complement** will unite with a definite amount of **amboceptor** and no more.

Amboceptor is specific in action—it will only attack the substance that stimulated its production.

Amboceptor only unites with **complement** in the presence of the substance which stimulated the production of the **amboceptor.** When **complement** unites with an **amboceptor** it can never leave that **amboceptor** to unite with another **amboceptor.** The amount of **amboceptor** per cubic centimeter in the serum of an animal depends upon the method of inoculation with the substance that stimulates **amboceptor** production, upon the size and number of inoculations and the interval between the time of inoculation and the time when serum is examined.

The amount of **amboceptor** per cubic centimeter in the serum of any given animal varies from time to time, usually growing less as the animal ages.

The amount of **amboceptor** per cubic centimeter in the serum of different animals of the same species subjected to identical inoculations is subject to wide variations.

When serum containing **amboceptor** is taken from an animal under aseptic precautions, placed in hermetically sealed containers and immediately stored in a refrigerator, the amount of **amboceptor** per cubic centimeter will remain almost constant, decreasing only a small fraction of a per cent. per month, for many months.

The addition of chloroform to serum to prevent bacterial growth has no effect on its **amboceptor** content. **Amboceptor** is not affected by heating to 55°C. for ½ hour; a temperature of about 60°C. or 70°C. will destroy it.

STANDARDIZATION OF AMBOCEPTOR

When performing complement fixation tests one adds 1 cc. of a 5 per cent. suspension of red blood cells to every tube, an amount of rabbit serum that contains sufficient amboceptor to insure complete hemolysis of these cells, with the amount of complement placed in the tubes, must also be added. As already stated rabbits treated in an identical manner give serum which varies in amboceptor content of immunized rabbit serum when one procures it. To do this a unit of measurement must be established and for this purpose the following rule has been established:

A unit of amboceptor is the smallest amount that will cause complete hemolysis of 1 cc. of a 5 per cent. suspension of red blood cells, together with 1 cc. of a 10 per cent. solution of fresh guinea-pig serum (complement), when incubated for 1 hour at 37°C.

Rabbit serum is not acceptable unless a **unit of amboceptor** is contained in less than 0.01 cc. of serum. The required accuracy of measurement cannot be accomplished, under ordinary circumstances, if one attempts to discharge from a graduated pipette less than 0.1 cc. of fluid—therefore, the first step in preparing amboceptor for standardization is to make a 1 per cent. solution of the rabbit serum in normal salt solution. To 9.9 cc. of normal salt solution add 0.1 cc. of **rabbit serum,** mix thoroughly.

Take a test-tube rack built to hold 12 tubes and place a tube in each of the first 10 holes and in the twelfth hole.

Put 0.1 cc. of the 1 per cent. solution of rabbit serum in the first tube.
Put 0.2 cc. of the 1 per cent. solution of rabbit serum in the second tube.
Put 0.3 cc. of the 1 per cent. solution of rabbit serum in the third tube.
Put 0.4 cc. of the 1 per cent. solution of rabbit serum in the fourth tube.
Put 0.5 cc. of the 1 per cent. solution of rabbit serum in the fifth tube.
Put 0.6 cc. of the 1 per cent. solution of rabbit serum in the sixth tube.
Put 0.7 cc. of the 1 per cent. solution of rabbit serum in the seventh tube.
Put 0.8 cc. of the 1 per cent. solution of rabbit serum in the eighth tube.
Put 0.9 cc. of the 1 per cent. solution of rabbit serum in the ninth tube.
Put 1.0 cc. of the 1 per cent. solution of rabbit serum in the tenth tube.
Do not put any rabbit serum in the last tube.
Put 1 cc. of the 10 per cent. solution of **guinea-pig serum in every tube.**
Put 1 cc. of the 5 per cent. suspension of red blood cells in every tube.
Shake the tubes to thoroughly mix their contents and place in incubator.

At the end of an hour remove and inspect the tubes, the appearance may be as follows:

The first one, two or three tubes may show little or no hemolysis; the next two or three tubes progressively increasing amounts of hemolysis and the rest, up to and including the tenth tube, complete hemolysis, the last tube no hemolysis.

Such an observation would establish the following: Absence of anything other than **complement** in the guinea-pig serum capable of hemolizing red cells as evidenced by the last tube.

The presence of complement in the guinea-pig serum, shown by the occurrence of complete hemolysis in the tubes containing a **unit or more of amboceptor**; that a sufficient number of tubes were run to determine the **unit of amboceptor**, because in addition to the tubes showing complete hemolysis there were others, containing smaller amounts of rabbit serum, in which hemolysis was incomplete, slight, or did not occur.

When a sensitized rabbit is bled from 10 to 20 cc. of serum is obtained, an amount sufficient for many hundreds of complement fixation tests. As the amboceptor content of this serum will remain practically constant, once the **unit** of a particular rabbit's serum has been determined, that quantity is taken as the measure with which to determine the **unit of complement** prior to the performance of each subsequent complement fixation test.

COMPLEMENT

Fresh unheated guinea-pig serum is ordinarily used to supply the **complement** for complement fixation tests. Guinea-pig serum is selected because it contains **complement** capable of uniting with amboceptors of either rabbit or human serum; because the average normal guinea-pig serum will not hemolize human or sheep red blood cells; guinea-pigs are comparatively easy to obtain, keep, handle and bleed.

The method of bleeding guinea-pigs is the same as described on page 231 for bleeding sensitized rabbits. After some practice one can save time and labor by withdrawing the blood from the pig's heart with a syringe. There are other advantages in so obtaining the blood: (1) When not more than 2 cc. of serum is required the withdrawal of the amount of blood necessary to yield the desired serum (about 5 cc.) can usually be accomplished without permanent injury to the pig. (2) For reasons to be explained later, it is better to use a mixture of the serum from several pigs rather than the serum of a single pig. (3) When not more than 5 cc. of blood is withdrawn from a pig, if an equal amount of normal salt solution is injected into the peritoneal cavity, the pig usually survives and several weeks later may be bled again. Some pigs survive as many as 10 bleedings.

When there is no particular hurry it is best to bleed pigs into Petri dishes, place the dishes in a refrigerator over night and collect the serum and make the complement fixation tests the following morning. In this way the maximum amount of serum is obtained with the least labor. To obtain the serum rapidly, bleed the pigs into centrifuge tubes, stir the blood with a glass rod and centrifugalize it at high speed for 5 or 10 minutes, the clear serum will rise to the top and can be at once pipetted off and used.

While the amount of **complement** per cc. in the serum of a guinea-pig is nearly constant, individual exceptions to the rule are met. The variations will be slightest when serum from several pigs is mixed. Although the serum of most guinea-pigs will not hemolize sheep or human red blood cells, occasionally a serum is obtained that does. For these reasons it is advisable to standardize **complement** before using it. The method of standardization is as follows:

Take a test-tube rack built to carry 12 tubes in a row and place a tube in each of the first 10 holes and one in the twelfth.

Make a 10 per cent. solution of fresh guinea-pig serum in normal salt solution by mixing 1 cc. of serum with 9 cc. of salt solution.

Put 0.3 cc. of the 10 per cent. solution of guinea-pig serum in the first tube.

Put 0.4 cc. of the 10 per cent. solution of guinea-pig serum in the second tube.

Put 0.5 cc. of the 10 per cent. solution of guinea-pig serum in the third tube.

Put 0.6 cc. of the 10 per cent. solution of guinea-pig serum in the fourth tube.

Put 0.7 cc. of the 10 per cent. solution of guinea-pig serum in the fifth tube.

Put 0.8 cc. of the 10 per cent. solution of guinea-pig serum in the sixth tube.

Put 0.9 cc. of the 10 per cent. solution of guinea-pig serum in the seventh tube.

Put 1.0 cc. of the 10 per cent. solution of guinea-pig serum in the eighth tube.

Put 1.1 cc. of the 10 per cent. solution of guinea-pig serum in the ninth tube.

Put 1.2 cc. of the 10 per cent. solution of guinea-pig serum in the tenth tube.

Put 1 cc. of the 10 per cent. solution of guinea-pig serum in the last tube.

Put one unit of **amboceptor** in every tube **except the last. Do not put any amboceptor in the last tube.**

Put 1 cc. of 5 per cent. suspension of red blood cells in every tube.

Shake the tubes to mix their contents and place in incubator.

At the end of an hour remove and inspect the tubes.

The last tube contains guinea-pig serum and blood cells only; if any hemolysis occurs in this tube it indicates that the guinea-pig serum is of itself destructive to blood cells and hence cannot be used in making complement fixation tests. The last tube should show **no hemolysis.**

Having learned from inspection of the last tube that the serum is acceptable, provided it contains **complement,** the other tubes are then inspected to detect the presence and quantity of **complement.**

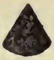
A unit of complement is the smallest amount that will cause complete hemolysis of 1 cc. of a 5 per cent. suspension of red cells, together with one unit amboceptor, when incubated at 37°C. for 1 hour.

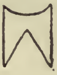
THE WASSERMANN TEST

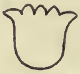
Just as normal untreated rabbits have no amboceptors in their blood serum which can act on red blood cells, normal humans who have not been infected with syphilis have no amboceptors in their blood serum which can act on syphilitic antigen.

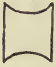
Just as rabbits that have been injected with red cells do have amboceptors in their serum which can act on red blood cells, humans who have been infected with syphilis do have amboceptors in their serum which can act on syphilitic antigen.

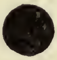
The Wassermann test is a test to determine the presence or absence of syphilitic amboceptors in a given individual's serum. For this purpose five substances are necessary:

1. **Syphilitic Antigen:** 

2. **Patient's Serum:** Contains no amboceptor, if negative.
   Syphilitic amboceptor,  if positive.

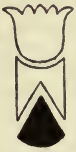
3. **Complement** (guinea-pigs): 

4. **Amboceptor** (immunized rabbits): 

5. **Red Blood Cells:** 

There are here four known substances with which to determine the presence or absence of a fifth, namely, syphilitic amboceptor =

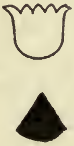
It is known that complement will join with amboceptor when that amboceptor's antigen is present; the complement is then fixed and can never join any other amboceptor. Hence, if the serum of a syphilitic person is placed in a tube with complement and syphilitic antigen and incubated for an hour the complement is fixed. There is no free complement in the tube.



Incubated at 37°C. for 1 hour.

Complement fixation.

If the serum of a non-syphilitic person is placed in a tube with complement and syphilitic antigen and incubated the complement is not fixed—the complement remains free and unaltered.



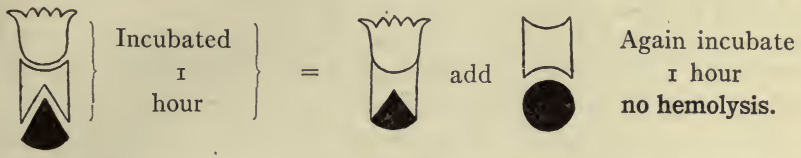
Incubated at 37°C. for 1 hour.

No Complement Fixation.

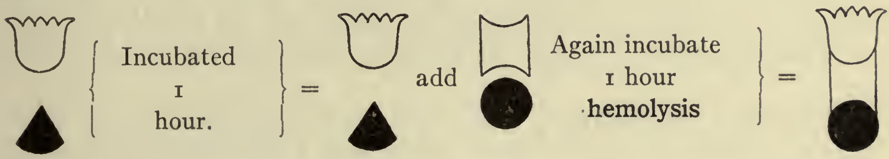
As placed in the tubes the complement, human serum, syphilitic antigen mixture is a clear, colorless fluid, almost like water in appearance. No change in its appearance occurs when complement is fixed, therefore, it is necessary to add something to these tubes that will indicate whether the complement remained free or was fixed. For this purpose, after the complement, human serum, syphilitic antigen mixture has been incubated for 1 hour, red blood cells and the inactivated serum of a rabbit sensitized against such cells are added.

And after shaking, the tubes are incubated a second time for 1 hour, then inspected.

If the complement was fixed during the first hour of incubation, then there was no free complement in the tube when the rabbit serum and red cells were added; since amboceptor alone cannot destroy antigen, the red cells remain unaltered, they precipitate to the bottom of the tube, and fluid above appears as water, there is no hemolysis. Such is the end appearance of a positive Wassermann test, indicative of syphilis.



If the complement was not fixed, remained free, during the first hour of incubation, then there was free complement in the tube when the rabbit serum and red cells were added. Since complement will join with amboceptor when that amboceptor's antigen is present and thereby form an antibody that will destroy the antigen, the red cells would be destroyed, the hemoglobin dissolved, the tubes show no sediment, but a clear, transparent red fluid hemolysis. Such is the end appearance of a negative Wassermann test—not indicative of syphilis.



PATIENTS' SERUM FOR THE WASSERMANN TEST

Only a fraction of a cc. of serum is required for a Wassermann test, an amount that can be obtained from 1, 2, 3 cc. of blood, but in all cases it is foolish to withdraw so small an amount. Frequently it is desirable to make the test in duplicate or triplicate; it is well to have an excess of serum that may be kept in storage in case one's findings are questioned, so it can be submitted to several independent workers for reexamination. It is necessary to have known negative and positive sera to control tests and they can only be accumulated by obtaining from patients more serum than is required for a single examination. No matter how skillful the operator, one does not always withdraw the full amount contemplated before operation. For these reasons it is best to withdraw from 5 to 10 cc. of blood. This blood may be obtained in several ways:

First.—Asepticize a finger, incise it and let the blood flow into a test-tube.

Second.—Asepticize an area over the most prominent vein of the forearm, thrust a needle into it and let the blood flow from the needle into a test-tube.

Third.—Have a sterile syringe attached to the needle that is thrust into the vein, withdraw the blood with the syringe and empty the syringe into a test-tube.

The third method is preferable because it is least dangerous to the patient and least painful. It does not require as large a needle as the second method and precludes soiling the clothes with blood and protects the withdrawn blood from contamination.

The first method is that of necessity and should never be resorted to when it is possible to obtain the blood by vein puncture.

However the blood is obtained the procedure must be made under aseptic conditions and guarded with the usual surgical precautions.

The blood is collected in a sterile test-tube at least ½ inch in diameter. If this tube is slanted as soon as the blood enters it, and allowed to remain at rest until the blood coagulates, then placed upright, clear serum will separate in several hours and can then be poured into another tube, inactivated and tested. If the tube must be kept upright from the time the blood enters it the serum is apt to be cloudy and should the interior of the tube be rough, the diameter less than ½ inch, serum may not separate from the clot until it is stirred with a glass rod, then it is always cloudy. When the serum is cloudy, has suspended matter in it, it must be centrifugalized until perfectly clear and free of suspended matter before testing. As a result of forceful expulsion of blood from a syringe into a tube, agitation during transportation or stirring with a glass rod, blood cells are ruptured and their hemoglobin tints the serum red, this discoloration of the serum cannot be removed, nor is there any need to; it does not affect the test. Serum that is sterile may be kept for weeks or months and still give the same reaction as when recently removed, but, for reasons that will be later explained, it is best to make the test within 48 hours after removal of the serum from the patient.

Occasionally, it is desirable to make a test as soon as possible, then the blood is collected in a centrifuge tube, gently stirred with a glass rod and centrifugalized at high speed for 5 minutes; in that time clear serum will collect at the top of the tube and can be at once removed, inactivated and tested.

The experience of the vast majority of serologists shows it is best to **inactivate** the patient's serum. This deprives it of complement. The serum should be inactivated immediately before testing and control sera should be inactivated every time they are used, regardless of how often that may be.

Sera are inactivated by placing the tubes containing them in a water bath at 55° or 56°C. for ½ hour. It will not matter if they are heated a few minutes more, but it is imperative that the temperature is maintained at 55°C. or 56°C.

Just as different rabbits injected with red blood cells have different amounts of amboceptor in their sera, different individuals infected with syphilis have different amounts of amboceptor in their sera. Wassermann, Neisser, Buchner, Morgenroth, Ehrlich and others discovered by experimentation that from 0.20 to 0.02 cc. of patient's serum might be used to make a complement fixation test for syphilis.

For reasons that will be discussed later, 0.1 cc. of patient's serum seems the ideal quantity to use, and in the Wassermann test as described in this book 0.1 cc. of the patient's serum is the amount always employed.

ANTIGEN FOR THE WASSERMANN TEST

Remembering that amboceptor does not fix complement unless the amboceptor's antigen is present, it is obvious that a syphilitic patient's serum, containing syphilitic amboceptors, cannot fix complement unless syphilitic antigen is present. Amboceptors are specific; they only act on the antigens that stimulated their production. Antigens are specific; they will only facilitate complement fixation when they act with their own amboceptors.

A priori, it would seem that the amboceptors of syphilis would be those formed to destroy the treponema pallidum, that they would be specific for the treponema pallidum and that the antigen for the Wassermann test would necessarily consist of treponema pallidum. This is not the case. Such amboceptors are present, in varying amounts in the majority of syphilitic sera. They usually constitute not more than 10 or 20 per cent. of the syphilitic amboceptors.

The amboceptors almost constantly present in syphilitic sera, the major amboceptor content of such sera, are specific for lipoid or lipoidal substance. Consequently the ideal antigen for the Wassermann test is composed of treponema pallidum and lipoid or lipoidal extracts combined. The next best antigen, and in most cases entirely sufficient, is a lipoidal extract without treponema. The least valuable of all is a pure culture of treponema.

Combined treponema and lipoid extracts are obtained by extracting the liver or liver and heart of a syphilitic fetus with alcohol. Lipoid antigens are alcoholic extracts or ether soluble-acetone insoluble extracts of the liver or liver and heart of a normal fetus or a normal guinea-pig; they may also be made from ox heart.

When the method of preparation is identical, different extracts vary in antigenic value, whether they be made from syphilitic fetal tissue, normal fetal tissue, guinea-pig tissue or ox heart.

The antigenic value of any extract can only be determined by testing it with a large number of known syphilitic sera and non-syphilitic sera, hence it is necessary in the beginning to procure from a reliable source an antigen of established value and standardized strength. The new antigens prepared by the beginner are then tested by repeated comparison with the original and when found to be equal to the original are stored for future use.

All these extracts possess two properties: **antigenic** and **anticomplementary.** The first is desirable, the second undesirable.

In this connection, we may define **antigenic** as the power of causing fixation of complement in the presence of syphilitic serum and in no other case; hence, will show no hemolysis with a syphilitic serum at the end of a Wassermann test, and show complete hemolysis at the end of a Wassermann test with all other sera.

Anticomplementary may be defined as the power of vitiating complement under any and all circumstances, when used in sufficient amount; hence showing no hemolysis at the end of a Wassermann test both with syphilitic and non-syphilitic sera.

When the smallest quantity of an extract that is anticomplementary is less than four times the mean antigenic quantity it must be rejected as unfit for use.

Titration shows that different quantities of an extract are antigenic, *e.g.*, from 0.01 to 1.0 cc. or from 0.1 to 0.5 cc., that is, in the first case, any quantity of the extract from 0.01 to 1.0 cc. would cause complement fixation with a syphilitic serum and have no effect on the complement with non-syphilitic sera; in the second case only quantities of extract from 0.1 to 0.5 cc. would do this. In the first case the **mean antigenic quantity** would be 0.5 cc.; in the second case the **mean antigenic quantity** would be 0.3 cc.

The **unit** of antigen, the amount used in making Wassermann tests, is the **mean antigenic quantity.** It is determined for each new antigen before use, and when in use antigen should be restandardized at least every month. The method of standardization will be described later.

CONTROLS

The end result and accuracy of any complement fixation test depends upon the purity and potency of the different substances used, their proper combination and quantities, the time and temperature of incubation. With so many sources of possible error it is imperative that each be controlled. Nine control tubes should be carried through with every Wassermann test and these tubes should always have the same positions in the test-tube rack. The author places them as illustrated in Fig. 1. Three of these tubes are used for making a Wassermann test, using a human serum that was obtained from a patient, who, so far as medical skill could determine, never had syphilis, a serum that previously showed a negative Wassermann reaction, a serum that does not in any way act on guinea-pig serum, rabbit serum, syphilitic antigen or red cells.

Three other controls are used for making a Wassermann test, using human serum that was obtained from a patient that presented typical positive clinical signs of syphilis, a serum that previously showed a positive Wassermann reaction, a serum that does not in any way act on guinea-pig serum, rabbit serum, syphilitic antigen or red cells, except that it fixes the complement in guinea-pig serum together with syphilitic antigen.

When a known positive serum gives a positive reaction, and a known negative serum gives a negative reaction, with the same reagents, in the same quantities, and under the same conditions as the unknown sera are subjected to, it is the strongest evidence that the tests have been properly performed and the results reliable—for this no other controls are necessary—but sometimes a test goes wrong, and these controls are not always sufficient to indicate the source of error; for this purpose three other controls are made: one for complement, one for hemolytic system and one for syphilitic antigen (Fig. 36).

Known positive and known negative sera, procured under aseptic conditions and kept sterile, if stored in a refrigerator, in all but very exceptional cases remain fit for use as controls for several weeks or months. They must be inactivated immediately before each time they are used; if this is not done they are not reliable.

For each serum that is to be subjected to the Wassermann test three tubes are used; one of these tubes during the first hour of incubation contains the suspected patient's serum and guinea-pig serum only, **no syphilitic antigen**; this is a control for the patient's serum. Only sera which contain nothing that can inhibit hemolysis, other than syphilitic amboceptors, are susceptible to examination by the complement fixation test. Since amboceptor cannot fix complement except in the presence of antigen, and there is no antigen in this tube, then the complement must remain free and unaltered, regardless of whether the serum is syphilitic or not.

If the complement in this tube is fixed or vitiated during the first hour of incubation it is evidence that the human serum contains something, not relative to syphilis, which makes impossible its examination.

In other words, the tube that contains no syphilitic antigen must always show **complete hemolysis** at the end of a Wassermann test; if it does not there is no diagnostic significance to the findings.

The writer has never seen a human serum cause this phenomenon when subjected to examination within 3 days after withdrawal from the circulation, but human sera, even when sterile, occasionally do so to a slight degree several weeks after they have been obtained from a patient; putrid sera nearly always vitiate complement. This control is therefore indispensable.

TECHNIQUE OF THE WASSERMANN TEST

1. Bleed patient or patients.

2. Compute the number of tubes that will be used in making the tests; allowing 0.1 cc. of guinea-pig serum for each tube, compute the amount required.

3. Obtain sufficient guinea-pig blood to yield 10 per cent. more than computed amount of serum required.

4. Collect blood in citrate solution to furnish red cells.

5. Wash and make a 5 per cent. suspension of the red cells in normal salt solution.

6. Make a 10 per cent. solution of the guinea-pig serum.

7. Make a 1 per cent. solution of sensitized rabbit serum.

8. Titrate or standardize the complement.

9. Separate patient's serum from clot and inactivate it.

10. Compute the amount of antigen required and mix the alcoholic extract with normal salt solution.

11. Place three tubes in the rack for each patient's serum to be tested, also three for the positive control serum and three for the negative control serum, place three tubes at the extreme right for complement control, hemolytic system control, and antigen control (see Fig. 36).

12. Put one unit of antigen in every tube in the middle and bottom rows except the hemolytic system control tube and the antigen control tube, put no antigen in the hemolytic system control tube, put two units of antigen in the antigen control tube, **do not put antigen in any tube in the top row.**

13. Put 0.1 cc. of patient's serum in each of the three tubes provided for it,

put 0.1 cc. of positive serum in each of the three tubes provided for it, put 0.1 cc. of negative serum in each of the three tubes provided for it.

14. Put one unit of complement in all the tubes in the top and middle rows and in the antigen control tube, put two units of complement in all the tubes in the bottom row, except the antigen control.

15. Shake each tube to thoroughly mix its contents and place in incubator at 37°C. for 1 hour.

16. Put two units of amboceptor (rabbit serum) in every tube except the complement control.

17. Put 1 cc. of the 5 per cent. suspension of red cells in every tube.

18. Shake each tube to thoroughly mix the contents and replace in incubator for 1 hour.

19. Inspect each tube and record appearance, place in refrigerator or allow to stand at room temperature for several hours and then make final inspection and record reactions.

INSPECTION OF THE COMPLETED WASSERMANN TEST

When the tubes are removed from the incubator at the end of the second hour of incubation the complement control tube should be inspected first. If it shows hemolysis the guinea-pig serum is at fault, the results are worthless and the test must be repeated with new guinea-pig serum. If it shows no hemolysis the hemolytic system control tube is next inspected; this should show complete hemolysis; if it does not an insufficient amount of complement or amboceptor has been used, the results are therefore worthless and the test must be repeated. The complement and hemolytic system controls presenting the proper appearance, inspection of the antigen control follows. Hemolysis should be complete; if it is not, an excessive quantity of antigen has been employed, the results are worthless, and the test must be repeated after restandardization of the antigen or the introduction of a new standardized antigen.

When complement control, hemolytic system control, and antigen control tubes all present the proper appearance, the known negative serum and known positive serum controls are inspected. The tube for each of these, containing no antigen, should show complete hemolysis. The other known negative tubes should both show complete hemolysis. The known positive tube containing one unit of complement should show no hemolysis, the other may show complete, incomplete or no hemolysis.

Only when all these controls are correct can one be confident that technical errors are absent (see Fig. 36).

Of the tubes containing the serum or sera under examination, those without syphilitic antigen are inspected first, they must show complete hemolysis, otherwise they are not susceptible to the Wassermann test.

Next in order is inspection of the tubes containing one unit of complement. If these tubes show complete hemolysis it is evidence of a **negative** reaction; in this case the tubes containing two units of complement will also show complete hemolysis.

When the tubes containing one unit of complement show no hemolysis the reaction is **positive,** indicative of syphilis; in this case the tubes containing two units of complement may show complete, incomplete or no hemolysis, dependent upon whether the 0.1 cc. of syphilitic serum contained just enough amboceptors to fix one unit of complement or more than that amount.

Occasionally, at the end of the second hour of incubation, half or more of the red cells will remain unhemolized in the tube containing one unit of complement. If incubated longer or allowed to stand at room temperature for 6 or 8 hours hemolysis may eventually be complete or it may remain incomplete. A serum which gives this atypical reaction may be syphilitic or it may not. Such a reaction must be reported **doubtful.**

PREPARATION OF ANTIGEN

Originally antigen for the Wassermann test was made by placing a finely hashed liver of a syphilitic fetus in a dark bottle with five times its volume of normal salt solution and shaking continuously for 48 hours. The contents of the bottle were then filtered through fine gauze and 0.25 per cent. of tricresol added. Such extracts are not as commonly employed at present because of the discovery that alcoholic extracts can be more easily made, are more stable and in the majority of cases are equally as good. Salt solution extracts frequently deteriorate so as to be useless in 1 or 2 months, some remain stable for years. It would seem from the known facts in regard to bacterial antigens in general that the salt solution extract of syphilitic tissue is the only extract that contains treponema pallidum antigen. Be that as it may, large series of comparative tests show that its only superiority is in some of the cases giving a doubtful reaction with other extracts.

Alcoholic extracts are the most commonly employed at present and are most easily made. The method of preparation is the same, whether syphilitic fetal liver, fetal heart, guinea-pig liver and heart or ox heart is used. The tissue is passed through a meat grinder or cut into as small pieces as possible with scissors, fibrous tissue removed and discarded, the rest ground to a pulp in a mortar and put in a ground-glass-stoppered bottle; 10 times its volume of absolute alcohol is added, the bottle hermetically sealed and shaken for 15 minutes. It is then placed in an incubator at 37°C. for 10 days, being shaken for 5 or 10 minutes every day. At the end of this time it is filtered through paper until perfectly clear and free of suspended particles and stored in a dark closet. Immediately before use it is diluted with salt solution, but as the salt solution dilution tends to deteriorate in several days or weeks, it is not good practice to dilute the alcoholic extract except as required.

Ether soluble acetone insoluble extracts, usually made from ox heart, have been very much lauded by some but in practice show little if any superiority over alcoholic extracts. The technique for preparing this antigen and its supposed superiority has been fully discussed by Noguchi (Proc. Soc. Exper. Med. and Biol., 1909, vii, 55).

Acetone soluble antigen does have some slight superiority over the others in that it tends to reduce to the minimum the occurrence of hemolytic and anti-complementary properties of the antigen. It is prepared by drying the ground-up tissue, *in vacuo*, over sulphuric acid, afterward placing it in acetone, 20 cc. for each gram of tissue. This is shaken for 5 minutes and incubated at room temperature, in the dark, for 10 days. It is then filtered through paper and the filtrate evaporated at 37°C. To each gram of the residue 100 cc. of methyl alcohol is added and it is stored at room temperature, protected from light. Immediately before use it is mixed with 10 times its volume of normal salt solution.

Any of the above antigens can be fortified by the addition of 0.25 Gm. of cholesterin to 100 cc.; this procedure seems undesirable as it is adding another reagent, unnecessarily, to a test already too complex.

Schurmann, Sachs, Rondin and others have employed various chemicals and colloids as antigen, but as yet no chemical antigen has been found to equal the tissue extracts. Varney and Baeslack have recommended an acetone extract of gummata removed from rabbit testicles following the successful inoculation of the rabbit testicles with treponema pallidum.

STANDARDIZATION OF ANTIGEN

For the standardization of antigen one requires at least 2 cc. of a known positive, syphilitic serum and a like amount of negative, non-syphilitic serum. Two rows of tubes are placed in a test-tube rack.

A 1:10 dilution of the alcoholic extract to be standardized is made by mixing 2 cc. of it with 18 cc. of normal salt solution.

This is put in the tubes as follows:

0.1 cc. in first tube in top row and first tube in bottom row.
0.2 cc. in second tube in top row and second tube in bottom row.
0.3 cc. in third tube in top row and third tube in bottom row.
0.4 cc. in fourth tube in top row and fourth tube in bottom row.
0.5 cc. in fifth tube in top row and fifth tube in bottom row.
0.6 cc. in sixth tube in top row and sixth tube in bottom row.
0.7 cc. in seventh tube in top row and seventh tube in bottom row.
0.8 cc. in eighth tube in top row and eighth tube in bottom row.
0.9 cc. in ninth tube in top row and ninth tube in bottom row.
1.0 cc. in tenth tube in top row and tenth tube in bottom row.
1.1 cc. in eleventh tube in top row and eleventh tube in bottom row.
1.2 cc. in twelfth tube in top row and twelfth tube in bottom row.

Next put 0.1 cc. of inactivated syphilitic serum in each tube at the bottom row; put 0.1 cc. of inactivated non-syphilitic serum in each tube of the top row.

Put one unit of complement in every tube (both rows). Shake each tube to thoroughly mix its contents and place in incubator.

When the tubes have been incubated exactly 1 hour, add one unit of amboceptor (rabbit serum) and 1 cc. of red cells to every tube, shake to mix contents and again incubate for 1 hour.

Should inspection reveal complete hemolysis in all the tubes in the top row, and no hemolysis in all the tubes in the bottom row, each of the various amounts of extract used are **antigenic** and the extract is apparently a good one. But these were not a sufficient number of tubes to show the smallest quantity that is antigenic nor the smallest quantity that is **anticomplementary,** hence the test must be extended.

Make a 1:100 dilution by mixing 0.2 cc. of the alcoholic extract with 19.8 cc. of normal salt solution.

Repeat the test exactly as before, except that the 1:100 dilution is used instead of the 1:10; this in practically all cases will show the smallest amount that is antigenic. Amounts of extract less than the minimum antigenic quantity will show complete hemolysis in the tubes containing non-syphilitic serum and will also show more or less hemolysis in tubes containing syphilitic serum.

The smallest **anticomplementary** quantity is yet to be determined; for this purpose repeat the test, using a 1:5 dilution of the alcoholic extract in quantities from 0.5 cc. in the first tubes to 1.6 in the last tube. Quantities that are anticomplementary show no hemolysis in the tubes containing syphilitic serum and incomplete, partial or no hemolysis in tubes containing non-syphilitic serum. The quantity of antigen to be used in making Wassermann tests—the unit of antigen—has been discussed on page 244.

When standardizing antigen, as a control, Wassermann tests are made on the human sera used in the standardization, and these tests are carried out with the antigen previously in use.

SPINAL FLUID

In certain cases it is desirable to subject the spinal fluid to the Wassermann test. The fluid is obtained when the patient is in bed, lying on his side with the body bent forward and thighs flexed. Under strict aseptic precautions (after local anesthesia if desired) a strong needle, at least 6 inches long, is thrust between the third and fourth lumbar vertebræ into the spinal canal. As the fluid comes out, drop by drop, it is caught in a test-tube until 5 cc. have been collected; the needle is then withdrawn, the puncture painted with tincture of iodine and covered with a sterile dressing. It is best for the patient to remain at rest in bed for at least several hours. In the absence of diseases other than syphilis the gross appearance of the spinal fluid is practically normal—clear, almost colorless and like water. Should it show any turbidity it is well, if not necessary, to centrifugalize it. Spinal fluid, like blood serum, should be protected from contamination, examined if possible within 48 hours after its removal from the patient, and inactivated by heating in a water bath at 55°C. for ½ hour before testing.

The technique is the same when making a Wassermann test with spinal fluid as with blood serum, except that six tubes are used each of which contains one unit of complement and the first two tubes each contain 0.1 cc. of spinal fluid, the third tube 0.2 cc. of spinal fluid, the fourth tube 0.3 cc. of spinal fluid, the fifth tube 0.4 cc. of spinal fluid, and the sixth tube 0.5 cc. of spinal fluid. The reason for this variation will be discussed in the following pages.

THE SIGNIFICANCE OF THE WASSERMANN REACTION

Probably no test has been subjected to a greater amount of investigation to detect and correct its faults and determine its value and proportionate weight in diagnosis. While there is much about it not understood at present and perhaps more to be learned, certain facts have been clearly established, which show this test to be of inestimable value.

There are factors, some of which are recognizable and removable, which at times cause the blood serum or spinal fluid of a patient infected with syphilis to give a negative Wassermann reaction. First among these to be considered is the stage of the disease:

In primary syphilis (from the appearance of the chancre until the appearance of the rash) the serum of most syphilitic patients gives a negative reaction in the first week, many give a negative in the second week, some give a negative result in the third, fourth and fifth weeks; nearly all give a positive reaction after this time.

In early secondary syphilis, when the disease is active, nearly 100 per cent. give a positive reaction; when the disease is latent from 5 to 20 per cent. give a negative reaction.

In tertiary syphilis, when active, from 1 to 10 per cent. give a negative reaction; when latent, from 20 to 25 per cent. give a negative reaction; when the reaction is negative in latent syphilis provocative treatment will frequently produce a positive reaction.

When the blood serum gives a negative reaction in late secondary and tertiary syphilis, the spinal fluid frequently gives a positive reaction.

In a small per cent., probably less than 2, of paresis both the blood serum and spinal fluid give a negative reaction.

The serum of a syphilitic patient, obtained during or shortly after the patient has been profoundly intoxicated with alcohol, will frequently give a negative reaction.

When a syphilitic patient has received sufficient mercury or salvarsan to partially or completely subdue signs and symptoms of pathological activity, even though the disease is destined to later manifest its presence, the reaction will frequently be negative for 3 weeks after the treatment has been stopped, occasionally negative for 6 weeks and rarely negative for longer periods, up to a year.

It is possible that some infections, secondary to syphilis, may cause a temporary or permanent suppression of a positive reaction. This has not been discovered but is mentioned as a possibility to be considered in certain cases and a lead to desirable research.

It is impossible to determine yet in what per cent. of dormant congenital syphilis the reaction is negative; the results so far obtained strongly indicate that it is small.

Nearly all cases of active, secondary and congenital syphilis give strong positive reactions when not influenced by medication or alcoholic intoxication. In latent or dormant cases of secondary and congenital syphilis, strong positive reactions are common, but taken as a whole they are less pronounced than in the active secondary stage; and in latent tertiary syphilis, the greatest number of weak positive reactions are observed; frequently the reactions are so slight that they must be considered doubtful.

As to the significance of a clear-cut positive reaction little need be said, yaws and leprosy are the only diseases other than syphilis that can cause a positive reaction; having excluded these, and technical error, a positive Wassermann practically always indicates syphilis.

The amount of amboceptor present in the serum of a syphilitic patient gradually increases from zero in the period of incubation, to many times the amount necessary to fix all the complement in 0.1 cc. of guinea-pig serum (using 0.1 cc. of the patient's serum) in the secondary stage of the disease. It tends to gradually decrease in the tertiary stage and may fluctuate as the disease is influenced by treatment.

As a consequence all degrees of reaction are observed in Wassermann tests, made with syphilitic sera, from no complement fixation to complete complement fixation.

With our present knowledge of the serology of syphilis there is little chance of error in interpreting the significance of a Wassermann reaction when all or none of the complement is fixed, but when very slight amounts of complement are fixed, when hemolysis is partial, not complete; in other words, when the reaction is doubtful, misinterpretations are inevitable. Fortunately, the patients giving such reactions are few in comparison to the whole; but they embrace a considerable number of syphilitics. Erroneous conclusions in these cases can be minimized by repeated examinations of the patient's serum at weekly or fortnightly intervals and making the tests with several different antigens and proportioning the reagents so as to make the test most sensitive—but error cannot be entirely precluded; herein lies the greatest weakness of the test.

Some apparently normal individuals and some non-syphilitic patients, especially those afflicted with any of the diseases classified as "chronic granulomata" have in their blood serum something which tends to deviate, vitiate or fix complement.

This non-specific deviation of complement can usually but not always be measured by placing 0.1 cc. of the suspected serum (inactivated) in each of a number of tubes, adding one unit of hemolytic amboceptor and one unit of red cells to each tube, and beginning with one unit of complement in the first tube add gradually increasing amounts of complement to successive tubes; shake, incubate for 1 hour and then discover the smallest amount of complement which gives complete hemolysis; the Wassermann test is then made, using this amount of complement as one unit.

Occasionally a serum that gives a doubtful reaction with a salt solution extract antigen will give a clear-cut negative or positive with an alcoholic or ether soluble-acetone insoluble or acetone soluble or cholesterinized antigen—or the reverse, so in doubtful cases repetition of the test with these different antigens may distinguish its true nature.

The employment of two units of hemolytic amboceptor is necessary in routine complement fixation to avoid error from non-specific deviation of complement. When a doubtful reaction is obtained if the test is repeated using only one unit of hemolytic amboceptor, after a preliminary examination of the suspected serum for non-specific complement deviation properties, a decided positive reaction is frequently obtained.

I have carefully studied this phase of the subject and believe accuracy is more nearly realized and the interests of patients best served by adhering to the following rule when the Wassermann reaction is doubtful:

If the patient has previously had unquestionable history, physical signs or serum reaction of syphilis, consider it positive.

If the patient's history contraindicated syphilis and there are no positive clinical evidences of the disease, eliminate the possibility of the reaction having been masked by mercury or arsenic or alcohol, give provocative treatment, obtain both blood and spinal fluid and examine both, using salt solution fetal liver extract, alcoholic extract and ether soluble-acetone insoluble antigens and two units of hemolytic amboceptor. If under these conditions more than half of the cells are hemolized in the tube containing 0.1 cc. of patient's serum and one unit of complement, and there is complete hemolysis in the tube containing two units of complement—reserve diagnosis and institute salvarsan treatment—if this does not alter the reaction, consider it negative.

It is the consensus of opinion that the Wassermann test is one of the greatest factors in medical diagnosis. Sporadically, prominent men attempt to belittle its value. He who will trouble to investigate will soon learn that the great majority of congenital syphilitics, unrecognized as such by physical examination are properly diagnosticated by this test; that in cases of doubtful nature, which if syphilitic are in the tertiary stage, the diagnosis without the Wassermann test is as often wrong as right, and with it the error is reduced one-half. These are not its greatest merits, but suffice to make it an indispensable part of every conscientious physician's armamentarium.

FURTHER CONSIDERATION OF THE WASSERMANN TEST

In the development of the complement fixation test for syphilis, Wassermann and his co-workers investigated each factor entering into it and the mechanism of reaction more thoroughly than any of their followers. In his original communication he states there is a fundamental reason for each step in the technique he describes and this has been amply proven.

Before the profession at large knew of the test several modifications were introduced by as many different experimenters. So many modifications have been recommended that it would require a large volume to tabulate them. The numerous modifications have one feature in common, namely, they have all failed to stand the test of time. Each has had its enthusiastic advocates but comparison with the original Wassermann, through long series of tests, has eventually established the superiority of the Wassermann test.

The experience of years of universal application of the Wassermann test has naturally amplified knowledge of the different elements entering into it and established certain facts that have permitted slight changes in technique, in no case basic, but such as to a slight degree simplify the performance of the test—as the introduction of alcohol, ether and acetone extracts of tissue for antigen, the reduction of the quantities used in the test to one-half the original volumes, the reduction of incubation from 2 hours to 1, and the introduction of quantitative determination of the syphilitic reaction.

The quantity of any one of the five substances entering into a complement fixation test must bear a proportionate relation, within certain limits, to all the others. Theoretically, the larger the quantity of patient's serum used the greater the possibility of detecting syphilitic antibodies when present in small amount. The amount of human serum that may be used is limited, however, by the irregular but frequent presence in human serum of substances, not related to syphilis, which tend to deviate complement. The proportion of human serum to other substances in the test as laid down by Wassermann is such that this non-specific deviation of complement cannot cause positive reaction in negative cases. Human serum also may contain substances which can hemolize red cells, without the presence or aid of complement; in the proportion of human serum to red cells used in the Wassermann test this effect is not observed, but if four or five times as much human serum is used it is frequently noted, this would give a negative reaction (hemolysis) even though the serum was positive, therefore, blood serum cannot be examined as spinal fluid is. When serum is not inactivated before subjecting it to a complement fixation test, a negative serum frequently shows a positive reaction; this is especially true when the antigen contains protein.

A reduction of the quantity of human serum used to make a Wassermann test necessitates a proportionate reduction in the quantity of all the other substances; the only advantage in such a procedure is a reduction in labor and expense which necessarily is associated with a greater probability of error, because no matter how careful a worker may be the smaller the quantities with which he works the greater the proportion of error in measurements; in addition to this, the Wassermann test seems to be a chemical reaction of colloidal nature and this further accentuates the danger of error in greatly reducing the quantities used in making the test.

A comparatively small amount of experimentation will convince one that human serum should be inactivated and that not less than 0.1 cc. nor more than 0.2 cc. should be used.

The sensitiveness of the Wassermann test, the power to show distinct positive reactions with human serum containing a very small quantity of specific amboceptor, can be increased by using amounts of antigen but slightly less than the minimum anticomplementary quantity.

The advantage so gained in obtaining positive reactions on syphilitic serum which otherwise would show a doubtful or negative reaction is more than offset by the frequency of spurious positive reactions, obtained with non-syphilitic sera by this method. This is the worst fault any test can have, hence it is unjustifiable to make the test more sensitive by increasing the amount of antigen used to more than one-fourth the minimum anticomplementary quantity.

Antigen, in whatever quantity employed, to some degree exerts an inhibiting effect on complement; human serum does likewise; when human serum and antigen are combined the total inhibiting effect is greater than the sum of both acting separately. The non-specific anticomplementary factor varies in the sera of different individuals. Within certain limitations, described as the Neisser-Wechsberg phase, a deficiency of complement can be made up for by an excess of amboceptor; therefore, if two units of amboceptor are used in the Wassermann test there is always sufficient compensation of the non-specific deviation of complement by human serum and antigen and false positive reactions are precluded. The disadvantage of this method of compensation is that frequently serum under examination contains not quite enough syphilitic amboceptor to fix one unit of complement and the anticomplementary factor of the human serum and antigen is low, so that the excess of amboceptor is sufficient to bring about a doubtful or negative reaction although the serum does contain a small amount of syphilitic amboceptor.

The temptation to increase the sensitiveness of the test by using one unit of amboceptor is too strong for many, but it is a baneful practice bound to elicit numerous pseudopositive reactions.

It is possible to measure the combined human serum and antigen non-specific deviation of complement; to do so is a procedure that consumes an amount of time and labor that forbids it as a routine procedure. In the comparatively few cases where the use of two units of amboceptor give unsatisfactory results and it is desirable to perform the test using one unit, the non-specific deviation of complement should first be measured and compensated for by the addition of the required amount of complement.

The selection of rabbit serum containing hemolytic amboceptor is worthy of note. In a previous chapter it has been stated that no serum should be used if more than 0.01 cc. was required to furnish a unit of amboceptor. It is not difficult to produce a serum 10 or 20 times as strong, so that 0.001 cc. contains a unit of amboceptor. The greater the amboceptor content the more desirable is the serum because, when a rabbit is injected with sheep cells, precipitins and agglutinins as well as lysins are formed; when the lysin (amboceptor) content is low the agglutinin and precipitin content is proportionately very much greater than when the lysin content is high; therefore, if a serum poor in lysins is employed precipitation and agglutination of the red cells occur and to some degree inhibit hemolysis. Furthermore, rabbit serum, like all other sera, may normally contain substances which tend to inhibit hemolysis and the possibility of such an occurrence is precluded when very small quantities are employed.

Generally the quantity of antibodies per cubic centimeter in serum is in proportion to the severity of an infection or the mass of inoculation; the greater the quantity of antigen inoculated, the greater the number and virulence of infective organisms, the greater the antibody production. Exceptions to this rule are numerous, especially and conspicuously so in syphilis. Any complement fixation test can be made quantitative as well as qualitative. Quantitative Wassermann tests are commonly made, usually four or five different degrees being distinguished; twenty or thirty different degrees instead of five might quite as well be recognized. The quantitative determination is made by adding different amounts of the patient's serum to a fixed amount of complement or different amounts of complement to a fixed amount of serum.

The majority of syphilitic individuals have sufficient amboceptors in 0.1 cc. of their serum to fix all the complement in the same amount of guinea-pig serum; many have more and some less. Citron recognized this fact early in the history of the test. The possibility of making the Wassermann test more sensitive by modifying the original technique and that given in previous chapters has been described—also the accompanying danger of obtaining pseudopositive reactions. If one believes it best to so conduct the test that a positive reaction will never be obtained with a non-syphilitic patient's serum, the quantitative determination is limited to distinguishing between sera containing slightly less than the average amount of specific amboceptors and those containing two, three or four times as much, or more.

It must be obvious to anyone familiar with the nature of the Wassermann test that an accurate quantitative reaction can only be obtained after a preliminary determination of non-specific complement deviation and compensation for same; this is practically never done in routine work—it cannot be—and therefore routine quantitative determinations are nothing more than rough estimates, indicating that the reaction is strong or weak as compared to the average reaction. It is desirable to recognize whether a reaction is weak or strong before treatment is instituted so that future tests will show whether the treatment has been effective or not, but this is of little real value because experience has shown that no treatment is permanently effective that does not cause a complete disappearance of specific amboceptors and hence a clear-cut negative reaction. I believe the practice of reporting reactions as +, ++, +++, ++++, is bad; it is implying a difference which is demonstrable serologically but not otherwise and it has led to a very general and totally erroneous opinion among physicians that there is a different pathological, therapeutic and prognostic significance according to the strength or weakness of the reaction. The Wassermann test, clinical and postmortem findings are in perfect harmony in showing that syphilis requires the most intensive treatment possible, medication to the patient's limit of toleration, whether the reaction be + or +++++; that it is frequently just as difficult, sometimes more difficult, to change a + to −, as to change a +++++ to −; that among the virulent cases that fail to respond to treatment and terminate fatally, many show a + or ++ throughout the disease and not a ++++.

There has been occasional mention in the literature of "Wassermann-fast" cases, patients having contracted syphilis and developed syphilitic antibodies which persisted in the blood undiminished by treatment and for long periods after cessation of treatment, after the disappearance of all signs and symptoms of the disease, persons apparently in good health and looked upon as having recovered. I have never discovered such a case; if they do occur they are very few. Every patient I have seen who persistently gave a positive Wassermann in spite of active treatment has had active syphilis, usually of the central nervous system, and with one exception all have died within 3 years after coming under observation.

COMPLEMENT FIXATION TESTS IN THE DIAGNOSIS OF INFECTIOUS DISEASES OTHER THAN SYPHILIS

In most, if not all, infectious diseases amboceptors capable of fixing complement in the presence of the offending organism are present in the blood serum. In most diseases other than syphilis the amboceptors are not so regularly or constantly present in sufficient amount to be demonstrable by a technique less sensitive than will elicit pseudopositive reactions, hence complement fixation tests in most diseases are not so valuable as other tests in establishing the nature of the infection. Probably future discoveries will greatly extend the field of usefulness of complement fixation tests.

Complement fixation tests may also be employed to determine the identity of an unknown antigen. No matter for what purpose this method of investigation is used, the principle is the same, the inherent non-specific properties of the substances entering into the test are the same, the danger of errors and the methods of precluding them are the same and consequently the technique should be the same.

Next to syphilis the complement fixation test is most frequently used in the diagnosis of gonococcus infections; not the acute suppurative infections of the eye, urethra or vagina, but in those cases where the heart, the Fallopian tubes, ovaries, testicles, seminal vesicles or the articulations are involved sometime after the subsidence of disease at the atrium of infection. In the absence of these complications or sequelæ the test is also made at times to determine whether or not the body is free of gonococci after the subsidence of gonorrhea and the disappearance of gonococci. The test has a distinct value, a positive reaction obtained 3 months after apparent recovery or during the course of an arthritis or endocarditis practically always indicating persistent infection, but it does not have so great a value as the Wassermann has in the diagnosis of syphilis, because a much larger number of persons infected with the gonococcus give a negative reaction.

In making a complement fixation test for gonococcus infection the technique is exactly the same as the Wassermann except, of course, gonococcus antigen not syphilitic antigen is used and the gonococcus antigen must be titrated immediately before each test.

Antigen for gonococcus complement fixation tests is made by culturing the gonococcus on Wertheim's or other appropriate media at 37°C. for 1 or 2 days, washing the growth off with normal salt solution, shaking the salt solution suspension of gonococci vigorously for 15 minutes or longer and adding 0.25 per cent. tricresol; keep in full dark containers in ice box.

The amount of salt solution used to wash off the growth and suspend it is such that the contained bacteria make it slightly cloudy or turbid. The method of standardization and titration is the same as previously described for Wassermann antigen except that one-half the minimum anticomplementary quantity is taken for the unit, the amount to be used in making tests. The value of a gonococcus antigen depends to a considerable degree upon its being polyvalent —including half a dozen or more strains of gonococci; this, together with the difficulty of culturing the organism, compels most serologists to depend upon some distributing laboratory for their supply. I have found the gonococcus antigens marked by Parke, Davis & Co., and H. K. Mulford Co. as good as any and superior to most.

The complement fixation test is a valuable aid in the diagnosis of echinococcus infestations; the filtered fluid from an echinoccus cyst, to which 0.25 per cent. of tricresol has been added, is used for antigen.

The detection of glanders and epidemic abortion of cattle is largely done by complement fixation tests.

Early discovery of active tuberculosis is one of the most important and difficult tasks of the physician. All available methods of investigating these cases are frequently inadequate. A test that can disclose the nature of the disease a short time after it becomes active, before serious permanent injury has been effected—and will indicate subsidence of the disease—will supply one of the greatest aids for further study and control of tuberculosis. The evidence accumulated during the last 5 years seems to indicate that such a test will be developed in the near future, and that it will be a complement fixation test. It has been well established that a demonstrable quantity of specific amboceptor is present in the blood serum of a large per cent. of patients in the early stages of pulmonary tuberculosis, a satisfactory antigen for routine clinical examinations has not yet been made, when this is done complement fixation tests in the diagnosis of tuberculosis will take a place as important as the Wassermann holds.

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The serum reactions described in this brief work are a very small portion of the subject of immunity. The best understanding of these reactions and their significance requires some knowledge of the subject in general.

Any attempt to present so complicated a subject to students, in a brief time, so they can master technique that will be available in diagnosis, must be to some degree dogmatic and, therefore, lacking the broadest discussions and presentation of facts desirable. With this in mind, the student inspired with true scientific thought is most earnestly solicited to read other books and monographs relative to the subject, especially those tabulated:

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CHAPTER IX

IMMUNITY

Immunity from disease is dependent upon innumerable extrinsic and intrinsic factors, many of which are not constants. Hence immunity is seldom if ever absolute, it is relative or conditional or partial; it varies in different geographical locations, in different individuals under similar circumstances; in each of us it ebbs and flows like the tide, changes from hour to hour and conspicuously varies at different age periods of life—in infancy, youth and old age.

We are more or less familiar with some of the extrinsic and intrinsic factors that establish and destroy immunity and there are others of which we know nothing.

Of all the ailments afflicting man half, or more, are infections—diseases in which the body is assaulted by microörganisms or their products.

The two most important factors in every case of infectious disease are first, the animal afflicted, and second, the offending microörganism. For infection to develop each of these factors must fulfill certain requirements and we may, for convenience of study at first, consider them separately.

PROPERTIES PECULIAR TO PATHOGENIC BACTERIA

Bacteria potentially capable of causing disease are those that produce aggressin, endotoxin, ectotoxin—one, two, or all of which can propagate in the host.

Aggressin.—Having inoculated animals with the typhoid bacillus and so produced a serous exudate, Bail collected this exudate, freed it of bacteria by centrifugalization and mixed a small quantity of it with a sublethal dose of typhoid bacilli. By injecting this mixture into experimental animals he produced death.

Bail's explanation of this phenomenon is substantially as follows: Pathogenic bacteria, upon entering a host, elaborate a substance that protects the bacterium and assures its development by paralyzing or destroying the bacteriocidal substances of the host which are attracted to the atrium of infection, especially those substances that effect phagocytosis.

This bacterial emanation, capable of inhibiting phagocytosis, he named aggressin.

Sterile serous exudates containing aggressin are of themselves poisonous; heating for 1 hour at 60°C. does not impair their toxicity; by repeated injections of small quantities of them a specific immunity to the aggressin and bacterium producing it can be conferred upon experimental animals.

Endotoxin.—By various experiments, not necessary to describe here, one can demonstrate that all pathogenic bacteria produce a water- and serum-soluble substance that is retained within the bacterium until it begins to involute or dies or disintegrates and is then liberated. Solutions of this substance, obtained by extracting dead bacteria with water or salt solution, when injected into animals produces some or all of the pathological changes and obvious signs of disease that are commonly produced by infection with the species of bacteria from which the soluble extract was obtained—this substance is referred to as intracellular toxin or endotoxin.

Endotoxins obtained from different species of bacteria differ in quantity, quality and resistance to heat. They all withstand heating to 55°C. for ½ hour, many withstand exposures of several hours to 60°C., 70°C., 80°C. or even 90°C.; a few, like tuberculin, withstand heating to 110°C. for several hours.

Repeated ascending doses of endotoxin obtained from certain species of bacteria confer immunity to the species from which the endotoxin was obtained—a specific immunity.

There is a close similarity in many respects between aggressin and endotoxin, in fact, Wassermann and Citron believe that what Bail described as aggressin is in reality endotoxin.

As a general rule, to which there are exceptions, the virulence of a bacterium is proportional to the nocuous power of its endotoxin.

Ectotoxin.—A few of the pathogenic bacteria throughout their active life emanate highly poisonous soluble substances that are absorbed by the tissue or medium upon which the bacteria are located. This poisonous emanation is referred to as extracellular toxin or ectotoxin. It is water-soluble and serum-soluble. The pathogenicity and virulence of bacteria that produce ectotoxin are largely dependent upon and proportional to the nocuousness of the emanation.

The ectotoxins as a whole are much less resistant to heat than endotoxins, the most stable being destroyed at 100°C. in a few minutes.

The ectotoxins produced by different species of bacteria differ widely in their affinity for and effect upon tissue cells *in vivo.*

Repeated injections of ectotoxin confer a relatively high degree of immunity to the specific ectotoxin injected and a slight immunity to the species of bacteria from whence it came.

If we study different strains of a single species or successive generations of a single strain, it soon becomes evident that bacteria vary in their toxin production; this is especially true of ectotoxin production. Slight alterations in temperature, aerobiosis, hydrogen ion concentration, acidity or alkalinity of environment, and pabulum, cause sudden, marked increases and decreases in ectotoxin production.

At times, for no apparent reason, cultures cease producing ectotoxin, the cessation being temporary or permanent. There are conspicuous exceptions as in the case of the diphtheria bacillus cultivated by Williams which has remained practically constant in its toxin production for years.

VIRULENCE

The virulence of bacteria is subject to alteration by many factors, some of which are known. Alterations in virulence may be temporary or permanent; both attenuation and exaltation of virulence occurs.

Pasteur showed that passage of rabies virus (which we assume is bacterial) through successive rabbits produces progressive alteration of its character and virulence up to a certain point, beyond which it remains constant, no matter how many times it is passed from rabbit to rabbit.

During its transitional stage, modification of the virus is shown by a gradual shortening of the period of incubation following inoculation, and curtailment of the stage of excitement when symptoms develop. When alterations in the character of the virus cease subsequent inoculations of rabbits are followed by a very uniform short period of incubation (5 to 6 days); the clinical signs of disease are those of a rapidly progressive and fatal paralysis without a preceding stage of excitement. Injected into animals other than the rabbit, or into man, fixed virus does not produce rabies—it confers immunity to rabies.

These observations may be summarized as follows:

First.—Passage of rabies virus from rabbit to rabbit produces alterations in it up to a certain point beyond which it remains constant in character and is referred to as "fixed virus."

Second.—Passage of rabies virus from rabbit to rabbit increases its virulence for rabbits and decreases its virulence for other animals.

If anthrax bacilli, primarily virulent to sheep, are cultivated at a constant temperature of 42°C. to 43°C., they gradually lose their virulence for animals in general. It is possible to carry this attenuation to any desired degree, and also possible to arrest it and thereafter maintain the strain at any desired virulence as is done in the preparation of anthrax vaccine (page 222).

In fixed rabies virus and anthrax vaccine we have two examples of permanent modification of virulence deliberately effected; the former being intensified for one species and attenuated for other species; the latter being attenuated for animals in general.

Among the various pathogenic species of bacteria strains are encountered that, from unknown causes, or as a result of prolonged cultivation on artificial media, have partially or entirely, and presumably, permanently lost virulence as in the case of the avirulent tubercle bacillus employed by Von Ruck to produce tuberculin.

From the evidence we have it seems highly probable that permanent alterations in virulence, both exaltation and attenuation, occur, but there is no criterion by which one can distinguish permanent from temporary alterations of virulence. Instances of presumably permanently attenuated organisms proving virulent are not lacking.

FLUCTUATIONS IN VIRULENCE

Much more commonly met with than permanent alterations of virulence are temporary alterations of brief duration. It is constantly observed in practice that most bacteria manifest their greatest virulence when passed directly from animal to animal, especially when passed from one animal to another of the same species, and show lessened virulence after periods of vegetative existence, particularly so if some of the conditions of environment have been detrimental to their development. Thus streptococci are observed which when isolated from a lesion of man are very virulent for rabbits and perhaps also virulent for guinea-pigs but after several weeks or months of cultivation on artificial media become avirulent for guinea-pigs and only slightly virulent for rabbits. If a large dose of such attenuated streptococci is injected into a rabbit, disease develops and organisms from the afflicted rabbit directly transferred to a second rabbit show increased virulence, and by several passages from rabbit to rabbit the original high degree of virulence may be restored.

Exactly what change occurs in bacteria that results in alteration of virulence is not clear, but some of the obvious changes manifested by some bacteria that have acquired immunity are suggestive.

ACQUIRED IMMUNITY OF BACTERIA

Normally rat blood is capable of destroying large numbers of anthrax bacilli. If a minute quantity of rat serum is added to a broth culture of anthrax bacilil and gradually increasing amounts are added to subcultures, eventually subcultures will grow abundantly in pure rat serum. This immunity is inherited by subcultures grown in plain broth and it is specific.

Danysz demonstrated that anthrax bacilli immune to rat serum have developed a mucous sheath or capsule. This mucous capsule unites with the germicidal substance in rat serum and makes it innocuous for anthrax bacilli.

Metchnikoff, Trommsdorf and others have shown that under analogous conditions streptococci, tubercle bacilli, the typhoid bacillus and cholera vibrio develop a mucous capsule which immunizes them against substances normally germicidal, including arsenic and bichloride of mercury; that many of these acquired immunities become permanent and are transmitted to future generations, that some, at least, of the bacteria retain all the faculties and virulence after acquiring immunity that they possessed before. Furthermore, the acquired immunity of bacteria is specific, *i.e.*, a bacterium that has acquired immunity to the serum of one species of animals is just as susceptible to the sera of other species of animals as it was before; anthrax bacilli that have acquired immunity to formaldehyde are just as susceptible to mercury and phenol as anthrax bacilli in general.

In acquiring active, specific immunity, some microörganisms, by the same process, acquire other new faculties. Effront has shown that when brewers' yeast is accustomed to living in media containing quantities of hydrofluoric acid germicidal to other yeasts, its power to produce alcohol is augmented and its susceptibility to chemical germicides other than hydrofluoric acid increased.

Although there is not sufficient data upon which to base broad generalizations, collected observations indicate that as a rule vegetative existence lessens and parasitic existence increases the virulence of bacteria; that when changed from a favorable to an unfavorable environment bacteria either lose some of their faculties or acquire new; virulence being exalted in some instances and attenuated in most; generally, when existence in an unfavorable medium is of short duration changes so effected are temporary, as manifested by a resumption of primary virulence when restored to a favorable environment; prolonged cultivation of bacteria in an unfavorable medium tends to produce permanent changes in them which become hereditary.

INFECTIOUSNESS OF BACTERIA

If we employ the word *infection* to indicate not merely the lodgment of bacteria upon or in tissue, but the subsequent multiplication of them with injury to the host, important differences in the infectious power of different species becomes apparent together with definite facts that determine in a given case whether the lodgment of bacteria upon or in tissue can or cannot initiate infection.

Webb has shown that the injection of one, two or three tubercle bacilli never produces tuberculosis; but the injection of 10 or more of the most virulent tubercle bacilli regularly produces tuberculosis. If tubercle bacilli only half as virulent are employed, however, the minimum number required to produce infection is more than 20. Webb made these observations when experimenting with monkeys.

There is a great mass of evidence indicating that the probability of infection following the lodgment of bacteria is in proportion to their number. It is highly improbable that infection ever follows the lodgment of one or several bacteria.

Certain organisms manifest a predilection for certain tissues, thus a quantity of rabies virus which will regularly produce disease when injected beneath the dura, very rarely causes infection when injected subcutaneously or into a muscle. Rapidly fatal, disseminated tuberculosis, as well as the chronic pulmonary type, rarely shows involvement of muscles.

The infectiousness of some bacteria is influenced by association or coincident lodgment with other species of bacteria and medicinal agents. When inunctions of mercury are applied at the point of entry within several hours after the lodgment of the treponema pallidum infection is frequently prevented. If quinine enters the body at the time tetanus spores gain lodgment, or shortly afterward, the development of infection is favored. Though the influences causing it are largely undeterminate, it is worthy of note in this connection, that the existence of diphtheria seems to predispose to scarlet fever; that diphtheria, superimposed on Vincent's angina, is unusually virulent; that the existence of gonorrhea favors secondary infection of the urethra by the pseudodiphtheria bacillus.

RESISTANCE OF MAN TO INFECTION

Resistance to infection is both general and specific. Man naturally possesses certain forces that tend to destroy any species of bacteria that gain lodgment upon or in the body. Many bacteria are destroyed by the hydrochloric acid of the stomach. Most pathogenic bacteria cannot cause disease until they have entered the body through the skin or mucous membrane and in a physiologically perfect state, the skin and mucous membranes are impervious to these organisms.

The blood contains fluids and solids that attack and attempt to remove or destroy any bacteria they meet. Most, if not all, the tissue cells of the body in a physiologically normal state to some degree resist invasion by bacteria.

In addition to these natural, general, immunizing forces, man acquires other, often stronger, specific resistance forces that act upon a single species of bacteria and are developed as a result of repeated trivial infections by that species or one acute infection by that species or treatment with a vaccine.

The effectiveness of both general and specific immunizing forces fluctuates with changes in the body state. It is proportional to the physiological condition and vigor of the body as a whole and to that of its various organs. When any of the body as a whole and to that of its various organs. When any of the body functions are impaired, arrested or destroyed, an immediate decline in resistance to infection occurs. Indigestion, insomnia and constipation all lessen resistance. Fatigue, mental or physical, lessens resistance. Of two extremities otherwise equal, differing in that one has been bruised or crushed—the injured extremity is most vulnerable to infection.

There is so slight a difference between the infectiousness of many bacteria and the corresponding resistance of the body, that frequently, the result of lodgment of pathogenic bacteria upon or in the body, whether they cause disease or are resisted, is determined by the state of the body at that time—exhausted or not exhausted, well nourished or poorly nourished, constipated or not.

Immunity, both natural and acquired, to some organisms is so nearly absolute that infection seldom follows exposure to it even when vitality is low and physiological activity and coördination poor.

MECHANISM OF IMMUNITY

Metchnikoff has clearly shown that many of the tissue cells and especially endothelial cells and polymorphonuclear leucocytes elaborate a ferment-like substance *Cytase* which destroys or facilitates the destruction of bacteria. Wright disclosed the presence in blood serum and fluid expressed from muscular and connective tissues of a substance called *opsonin* which facilitates ingestion and digestion of bacteria by phagocytic cells. Pfeiffer discovered that under certain conditions peritoneal exudate causes a granular degeneration of the cholera vibrio.

PFEIFFER'S PHENOMENON

Pfeiffer's test (page 123) is explained, on experimental evidence, by Metchnikoff as follows:

Immediately after the injection of living cholera vibriones into the peritoneal cavity of a guinea-pig, disintegration of the phagocytic cells surrounding the organisms occurs—**Phagolysis**—with a consequent liberation of their cytase. The cytase liberated by the cells of susceptible animals is not able to injure the cholera vibriones and consequently they retain their original morphology and motility and multiply.

The phagocytic cells of immunized guinea-pigs contain a more potent cytase and when this is liberated by phagolysis it causes the granular degeneration and loss of motility and arrest of propagation observed.

Bordet observed that when a sublethal dose of streptococci is injected into the peritoneal cavity of a guinea-pig a high leucocytosis promptly occurs and the cocci are all taken up and destroyed by the leucocytes. If a lethal dose is injected phagocytosis occurs as before but some cocci escape and these produce a generation more resistant to phagocytosis so that, even though leucocytosis continues high a greater proportion of this second generation escapes destruction, the cocci can multiply faster than the phagocytes can destroy them and general dissemination results.

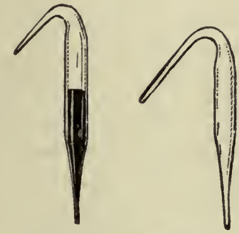

FIG. 43.—(*Citron.*)

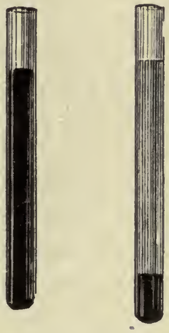

FIG. 44.—(*Citron.*)

In his original investigations of phagocytosis Wright devised a method of studying changes in it, now known as the **opsonic index test**. The test is made as follows:

Blood is collected in a capillary tube from the patient, the tube is sealed and put aside to clot (see Fig. 43). Several loopsful of bacteria are removed from a 24- or 48-hour-old culture on solid media and emulsified with normal salt solution; this suspension is drawn into a tube and centrifugalized for a minute to throw down clumps; the supernatant suspension is then removed. Blood is collected from the patient's finger in a capillary tube, half filled with 1 per cent. sodium citrate in normal salt solution. The tube is shaken to mix the citrate solution and blood, sealed, and centrifugalized until a distinct layer of white cells is seen upon the top of the rods. The white cells are pipetted off and placed in a sterile watch crystal. The patient's serum is poured from the clot into another sterile watch crystal and the bacterial suspension is placed in a third watch crystal.

The bulb of a mixing pipette is compressed and one volume of white cells drawn in by partially releasing the bulb; the bulb is further released so that the cells retreat from the tip; then a volume of bacteria is drawn into the tube and a bubble of air admitted; finally a volume of patient's serum is taken into the tube.

The contents of the tube are then thoroughly mixed by ejecting them into a sterile watch crystal and drawing them back into the tube several times. When mixed the fluid is drawn to the middle of the tube, the ends sealed and an identification mark placed on the tube. The tube is then incubated at body temperature for 15 minutes in an incubator that will permit frequent rotation of the tube as this is necessary (see Fig. 45).

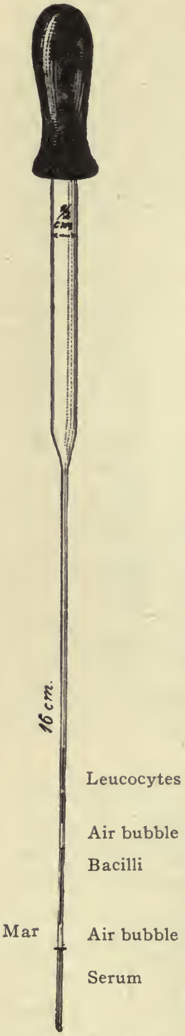

Fig. 45.—(*Citron.*)

At the same time that blood is drawn from the patient to obtain serum, blood is also collected for the same purpose from several healthy persons and the normal sera is pooled (mixed).

A second mixing pipette is loaded with equal volumes of cells, bacteria and pooled serum and treated exactly the same as the tubes containing patient's serum.

When the period of incubation is up, the tube containing patient's serum is emptied on a clean slide, one drop of the fluid is transferred to each of several other slides and these drops are spread in a thin film.

The slides are fixed and stained. Staining with Leishman's blood stain is the common method but primary fixation with methyl alcohol for 1 minute followed by staining with dilute fuchsin for a minute gives a more distinct field. The slides so prepared are examined with the oil immersion lens. The number of bacteria within each leucocyte is recorded and when 100 leucocytes have been examined the average number of bacteria per leucocyte is computed; this number is the patient's **opsonic content.**

By examining in the same way the contents of the second tube the normal opsonic content is determined.

The patient's opsonic content, divided by the normal opsonic content, equals the patient's **opsonic index.**

Using this test Wright discovered the following facts. When washed free of blood serum leucotyes almost entirely lose their phagocytic power; in other words, there is some substance in blood serum which does not destroy bacteria but acts on them so as to facilitate their ingestion and digestion by leucocytes; to this substance he gave the name *opsonin.* The quantity of potency of opsonin varies; it is low when immunity is slight and high when immunity is strong. There is natural opsonin—that which is present throughout life regardless of infection—and acquired opsonin, developed as a result of infection or vaccination.

There may be both thermolabile and thermostabile opsonins present at the same time in a given animal's serum.

Opsonin seems to be entirely specific in nature—in an animal it may be below normal for one organism and normal for other organisms; *in vitro,* the opsonin for one species of bacteria may be exhausted from a serum without lessening in any way the opsonic content for other species of bacteria.

When a patient suffers infection his opsonic content is primarily below normal for the infecting organism; if the disease progresses to a favorable termination the opsonic content of the patient's serum, for the infecting organism, shows a progressive increase from shortly before or after the disease reaches its fastigium until recovery, and it may be distinctly above normal in the period of convalescence—continuing so for weeks or months.

Vaccines produce a similar effect. Following the administration of a dose of bacteria or bacterial extractives there is a sharp fall in the opsonic content, the degree and duration of which is proportioned to the dose; this period of depression is known as the "negative phase."

An overdose (poisonous dose) of vaccine frequently induces a negative phase of long duration followed by a slow return to normal. In a few favorable instances an overdose is followed by a marked fall of short duration superseded by a rapid elevation above normal. Therapeutic or immunizing doses produce a slight negative phase of 12 to 72 hours duration followed by a gradual rise, 2 to 12 days in duration, averaging a distinct elevation of the opsonic content above that which existed prior to treatment.

Slight fluctuations in the opsonic content for the infecting organism during the course of a disease frequently are not associated with discernible changes in physical signs but marked fluctuations usually are, descent of the opsonic content being associated with physical signs of ill omen and ascent concomitant with favorable changes in physical signs.

OTHER PROTECTIVE SUBSTANCES IN BLOOD SERUM

Previous descriptions of agglutinins, lysins and antitoxins explain in part the stimuli causing production of such of these as are acquired, their degrees of specificity and mode of action and their more important physical properties and effects.

There are specific agglutinins, lysins, precipitins and antitoxins present in the blood serum prior to infection or inoculation. These form part of the natural protective armament against infection. In general, primary antibodies are less abundant and less effective than those formed as a result of infection or inoculation (acquired antibodies) but are identical in nature and action.

There are notable exceptions to this rule as evidenced by immunity to diphtheria. Schick and Park have shown that about 75 per cent. of all children are immune to diphtheria during the first 3 months of life; during the remainder of the first 2 years only 50 per cent. are immune; the percentage of children immune to diphtheria gradually increases from the second year of life up to maturity, at which time about 80 per cent. are immune even though they have not had the disease. Evidence indicates that this immunity is largely, if not entirely, due to the presence of antitoxin in the blood. Here then is an example of natural antitoxin present in sufficient quantity to protect against infection;

antitoxin received from the mother before birth and lost during the first 3 months of life but gradually replaced by the possessor's own cells and apparently without the stimulus of infection.

Rabbits normally possess agglutinins for pneumococci but after inoculation with sublethal doses of pneumococci they develop more abundant, and, therefore, more effective pneumococcus—agglutinin. Bull has found that pneumococci injected intravenously soon disappear from the circulation as a result of agglutination. If sufficient numbers are injected the natural agglutinin is exhausted and cocci escaping destruction lodge in tissue and multiply. After a time, stimulated by the infection, more agglutinin is produced and coincident with the appearance of acquired agglutinin recession of the disease begins.

In addition to the antibodies that have been demonstrated and described, there are others, possibly of equal importance in the establishment of immunity, that are still obscure. Sclavo's serum which unquestionably possesses immunizing power contains little or no opsonin, complement, lysin, agglutinin, antitoxin, precipitin or cytase. It is observed at times that serum containing no demonstrable antibodies from an immune animal will prevent the multiplication of bacteria without otherwise affecting them.

THEORIES OF IMMUNITY

After more than a quarter of a century of research and observation of the phenomena accompanying infection and immunity in various animals from the simple unicellular forms up to and including man, Metchnikoff concluded that invading organisms exert varying degrees of chemiotaxis; some a negative chemiotaxis, others a slight positive and some a strong positive chemiotaxis on the phagocytic cells of the host. In the mammals this action is exerted on certain body cells and is most strikingly exemplified by the chemiotaxis of some bacteria for leucocytes.

He believed the production of cytase and other antibodies, above and in addition to what normally exists, depends upon two factors: stimulation of the cells of the host by the invading organism; and an increased production of cytase, agglutinin, lysin, etc., by the cells stimulated, the increase being in quantity or quality or both.

Metchnikoff considered digestion of bacteria within the phagocytic cells of the body the ultimate and also the essential process in ridding the body of bacteria. He believed excretory organs in a normal condition never excrete bacteria. He assumed that the action of antibodies in the blood serum on bacteria facilitates their ingestion and digestion by phagocytic cells but considered bacteriolysis an exclusively intracellular phenomenon.

The observations of some of his contemporaries and followers have amply confirmed some of Metchnikoff's views and discredited others. It is now recognized that the establishment and maintenance of active immunity and the conquest of infection is a matter of cellular activity; in some cases the activity of phagocytic cells and in other cases the activity of cells (not phagocytes) that emanate antibodies which flow in the blood serum and attack, arrest or destroy bacteria or neutralize their toxins.

EHRLICH'S THEORY

From his studies of the production of antitoxins, agglutinins, lysins and precipitins and their action both *in vivo* and *in vitro* Ehrlich evolved a theory of immunity commonly referred to as "Ehrlich's Side-Chain Theory," which may be briefly summarized as follows:

The digestion of food in the alimentary canal is not an adequate preparation of it for assimilation by various tissue cells, hence each tissue cell of the body must select and draw to itself certain substances in the blood serum and further digest them for its nutrition; and reject other substances in the serum not appropriate to its needs.

Tissue cells possess numerous side chains, each having an affinity for a particular substance needed by the cell for its nutrition.

In conformity with a general law of nature when these side chains or receptors are exhausted and when the requirements of the cell increase, a superabundance of new receptors is produced. Receptors produced in excess of the cell's needs are cast off and circulate in the blood serum. Receptors in the blood serum manifest the same specific affinities and unite with the same substances as when attached to cells, precipitating, agglutinating, neutralizing or disintegrating them. This may be illustrated by the following diagrams (Fig. 46.):

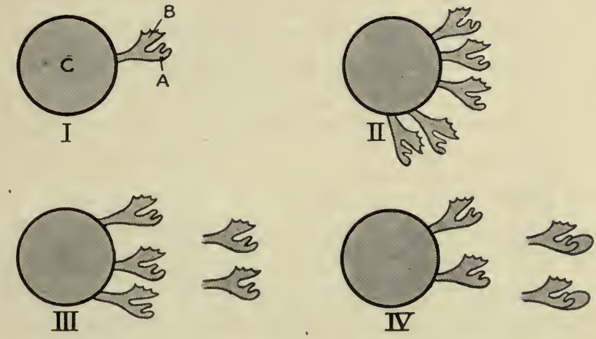

FIG. 46.

I. Represents a cell with two different kinds of receptors. *C*, cell; *A*, receptors with affinity for one substance; *B*, receptors for another substance.

II. Represents the same cell after stimulation producing an increased number of receptors.

III. Some of the excess receptors cast off by the cell.

IV. Represents poisonous substance or food with which receptors *A* would unite.

Appetite, digestion within the stomach and intestines and absorption from these organs is for alimentation; some substances devoid of nutritive properties are not assimilated when taken into the alimentary canal, but pass through it without affecting the body.

One may possess an appetite for that which is poison and not food; the stomach and intestines may digest and pass into the circulation some noxious substances. The same is true of cellular digestion. Some cells possess receptors which manifest an equal or greater affinity for certain poisons than for food; if the blood serum brings such poison to a cell so equipped it is assimilated and as a result one of two possibilities result: the cell dies, or it is injured, the receptors uniting with the poison exhausted, the cell temporarily arrested in its activities; the arrest stimulates greater activity on the part of the cell, and as it recuperates the cell not only reproduces the destroyed parts, but forms many more receptors than were exhausted, so many, that some are cast off and taken up by the blood serum. If the blood serum receives more of the poison, the free receptors in the serum unite with it and precipitate, agglutinate, neutralize or disintegrate it, so preventing the poison from reaching and injuring the cell—the cell is immune so long as the serum contains free receptors.

Ehrlich showed that these receptors or antibodies are divisible into three groups: receptors of the first order, receptors of the second order and receptors of the third order. There are certain properties common to all three and others peculiar to each group. All are products of cellular activity, all occur in blood serum, all are to a degree specific in action uniting with a single substance, all may be increased up to a certain point by stimulation of the cells producing them.

Receptors of the first order include the antitoxins. In the blood serum they are almost or entirely confined to the pseudoglobulin portion. They are relatively stable, serum containing them showing a loss of about 1 per cent. or less of its receptor content, for months after removal from the body, if kept sterile and in a cool, dark place (ice box). An exposure of 60°C. to 80°C. for ½ hour destroys them.

Receptors of the second order include the agglutinins and precipitins and these are more complex than receptors of the first order having two distinct parts, one part which unites with the substance to be agglutinated and another part that produces the agglutination. Receptors of the second order remain active in serum for months after removal from the body if kept cool and sterile. An exposure of ½ hour to 70°C. permanently destroys receptors of the second order.

Receptors of the third order, the most complex of all, include the lysins and have been previously described (page 267).

DISCUSSION OF THEORIES OF IMMUNITY

The theories of Metchnikoff and of Ehrlich both recognize that active immunity is dependent upon cellular activity, and exaltation of it is the result of stimulation (irritation) of the cells by the poisonous substance against which immunity is enhanced.

The recognizable mechanism of infection and immunity amply substantiate this.

Numerous observations indicate that the mechanism of infection, resistance and immunity is in some cases substantially as explained by Ehrlich; in others as described by Metchnikoff and Wright, but in many instances neither of these hypotheses harmonize with the facts.

The method of attack of different groups of bacteria varies, the results of their assaults vary and the consequent defence and resistance of the host vary to combat these dissimilar infections.

Thus we observe in diphtheria no invasion by the bacteria but intoxication with extracellular toxin and this is combated largely if not entirely by antitoxin.

In typhoid and paratyphoid infections, leucocytosis is conspicuous by its absence; convalescent and immune persons having agglutinins, precipitins and lysins in their blood serum in greater amount than before, in greater amount than is generally observed in the serum of persons not immune to typhoid infection.

In acute, localized staphylococcus and streptococcus infections, leucocytosis and phagocytosis is marked; the convalescent patient's serum contains practically no antitoxin, little if any precipitin, agglutinin or lysin, but does contain an increased amount of opsonin.

After infection or treatment with dead bacteria or bacterial products the result is not always immunity or exalted resistance. Some species of bacteria may infect one time after time apparently leaving the host more susceptible to subsequent infection than he was primarily, suggesting that cellular reaction as described by Ehrlich and Metchnikoff does not always occur and that recovery is possible without it.

ANAPHYLAXIS

Metchnikoff observed in unicellular organisms that phagocytosis was not confined to invading parasites and food; inanimate poisons frequently being disposed of in the same way. Also in the study of agglutinin, precipitin and lysin production, Ehrlich and his co-workers discovered that such antibodies were generated by the injection of many inanimate proteins.

Using the term **antigen** (haptin) to designate a substance which when introduced into an animal is a foreign irritant, or splits into simpler compounds some of which are irritant or toxic, and stimulates the cells of the injected animal to produce specific agglutinins, precipitins, antitoxins, lysins, or other antibodies or specific ferments, and as a result makes the injected animal either immune or hypersusceptible to subsequent injections, we find a great variety of proteins in common animal and vegetable matter and in foods, act as antigens.

As to why the injection of dead typhoid bacilli (antigen) or infection with living typhoid bacilli (antigen) *immunizes* against future invasion by typhoid bacilli, and injection or ingestion of red blood cells from another species (antigen) or blood serum from another species (antigen) or egg albumin (antigen) creates a *hypersensitiveness* to the injurious effect of similar subsequent injections of these substances is a matter of speculation.

This sensitization by foreign proteins is a serious phenomenon of common occurrence.

When produced by bacteria or bacterial products it is referred to as **allergy.**

When produced by other substances it is referred to as **anaphylaxis.**

As commonly thought of, allergy or anaphylaxis is a state of hypersensitiveness to a foreign protein, produced by the primary dose of that protein and manifest only after a second dose.

This phenomenon was first observed by Theobald Smith when studying the effect of repeated subcutaneous injections of horse serum into guinea-pigs. He found that in practically all cases the first injection of horse serum into a guinea-pig is innocuous; the animal shows no subsequent discomfort, illness or injury. A second injection, somewhat less in quantity, equal to, or greater than the first, in a few minutes, always less than 10, and sometimes almost instantly, produces signs of great distress and usually death.

The animal shows difficulty in breathing, air hunger, throws up its head falls paralyzed and expires. Occasionally the animal does not die but lies on its side breathing rapidly for several minutes or hours and then quickly recovers completely.

Guinea-pigs that have manifested these signs of hypersensitiveness and have recovered are thereafter non-sensitive, a subsequent injection or injections of horse serum causes no illness or injury—the animals are immune.

The state of hypersensitiveness does not develop immediately after the first injection; it develops gradually, does not reach its fastigium until 2 or 3 weeks after the first injection and may be deferred. There is a minimum quantity of horse serum which will produce anaphylaxis and larger quantities having the same effect. The larger the quantity used for the first injection the longer the interval before hypersensitiveness develops.

During the period of incubation—the interim between the first injection and the development of hypersensitiveness—a second injection causes no disturbance, but somewhat lengthens the period of incubation beyond what it otherwise would be. Multiple injections during the period of incubation have the same effect. A guinea-pig sensitized to horse serum continues hypersensitive to it throughout life unless immunized, so that a second injection given a year after the first will be followed by a typical anaphylactic attack.

As small a quantity as 1/1000 cc. of horse serum subcutaneously has caused a fatal reaction in sensitized guinea-pigs and 1/50,000,000 cc. of pollen extract has induced a violent attack of hay fever.

Many investigators throughout the world have confirmed the observations of Smith.

Recent studies have shown that anaphylaxis, like immunity, may be passive or active, passed from mother to progeny or acquired, and may be due to the blood serum alone, fixed tissue cells alone or both.

The phenomena of anaphylaxis and allergy—sensitization by a primary non-toxic dose after a period of incubation, sudden onset of respiratory disturbance and paralysis following a second injection and terminating in death or complete recovery with subsequent immunity—has been produced in guinea-pigs and other animals with extract of oats, egg albumin, other proteins and bacteria.

From these observations and certain similarities to what has been well known as idiosyncrasy, it has been assumed by some that anaphylaxis, allergy and idiosyncrasy are essentially the same. This may or may not be true.

There are two apparent differences between idiosyncrasy and allergy or anaphylaxis: idiosyncrasy is a hypersensitiveness disclosed by a *primary* dose of the irritant; this hypersensitiveness *persists* after recovery from one or more doses that precipitate symptoms of intoxication.

Those who hold the opinion that anaphylaxis, allergy and idiosyncrasy are basically the same, look upon luetin, mallein, typhoidin and tuberculin reactions as anaphylactic manifestations. Until more is known it is unsafe to generalize.

Sensitization to tuberculin may be general or localized. An animal afflicted with active tuberculosis causing general toxemia shows a generalized hypersensitiveness to tuberculin; should the disease become dormant, hypersensitiveness may continue at the same degree as when the disease was active or it may be reduced; or, general sensitization may decline, but certain organs or tissues, as the skin and conjunctiva, continue equally as sensitive in the dormant state of the disease as they were in the active state.

Local sensitization to tuberculin without generalized sensitization can be produced in non-tuberculous animals. The instillation of tuberculin will sensitize the conjunctiva of a healthy rabbit's or man's eye so that a second instillation causes a violent local reaction, while a primary instillation in the opposite eye, at the time of the second instillation in the sensitized eye, produces no reaction.

The recent investigations of Manwaring and Kusama show that in some instances, at least, a dose of antigen administered to a sensitized animal immediately acts on the lungs in a manner that greatly impairs or totally prevents respiration and aeration of the blood. While such pathological changes account for the respiratory distress commonly observed in anaphylactic attacks and could be responsible for sudden death, they do not explain the paralysis. Other investigators have shown involvement of other vital organs, especially the kidneys.

By perfusing the lung of a normal non-sensitized animal, with a mixture of blood serum and antigen from a sensitized animal, the same pathological changes are produced as occur in a sensitized animal following a dose of antigen.

Manwaring and Kusama state that the serum of a guinea-pig immune to horse serum, when mixed with horse serum makes it inert just as diphtheria antitoxin neutralizes diphtheria toxin, so that the injection of a mixture of immune guinea-pig serum and horse serum into a sensitized guinea-pig does not produce any manifestation of anaphylaxis. They also report that a guinea-pig immunized to horse serum enjoys immunity by virtue of substances in its blood serum which act on horse serum and neutralize it before it reaches fixed tissue cells. The fixed tissue cells of such immune animals are hypersensitive to horse serum; this being shown by freezing the lungs of an immune guinea-pig of their native blood and perfusing them with a mixture of normal guinea-pig serum and horse serum, the perfusion causing a typical anaphylactic manifestation.

It is a natural and common tendency to explain anaphylaxis so as to harmonize it with Ehrlich's theory of immunity: assuming that the first injection or ingestion of antigen does not produce ill effect because it is not toxic as taken in, only becoming toxic when disintegrated; at the time the body receives its first dose of antigen the body cells and fluids are poor in ferments or receptors or both, capable of disintegrating the antigen, hence it is slowly disintegrated, the toxic substance being slowly liberated in quantities too small to irritate; this antigen must be disintegrated (to feed the body or be eliminated) and its presence is a stimulus to the body cells to produce much more ferment and receptors capable of disintegrating antigen and hence capable of liberating its toxic components; this stimulated new activity of the body cells results in a gradual increase of ferments and receptors, the quantity necessary to disintegrate antigen so rapidly as to liberate at once sufficient toxin to produce obvious injury, does not develop or accumulate until one or more weeks after the stimulus to production caused by the first dose of antigen; a second injection of antigen given in the early days of increased antibody production (period of incubation) does not produce ill effect for the same reason that the primary does not; a second injection of antigen given when antibody production has reached its maximum (period of hypersensitiveness) produces ill effect because it is rapidly disintegrated and therefore an injurious quantity of toxin suddenly liberated; animals that survive this injury, by it have their cells stimulated to form receptors or ferments for the toxic component liberated when antigen is disintegrated, hence subsequent doses of antigen do not produce ill effect (the animal has lost its hypersensitiveness, is immune) because there are antibodies in the animal's blood serum that neutralize the toxin as fast as it is liberated by disintegration of antigen. This explanation is largely hypothetical.

RELATIONSHIP OF ALLERGY, ANAPHYLAXIS AND IDIOSYNCRASY TO IMMUNITY

In a previous chapter immunity was described as a relative, not absolute, state subject to fluctuations; equally dependent upon intrinsic and extrinsic factors.

Susceptibility and hypersusceptibility (hypersensitiveness) are also relative conditions subject to changes and dependent upon both intrinsic and extrinsic factors. Furthermore, it is observed that in many instances the same factors that are active in producing immunity are also active in producing allergy and anaphylaxis. All the demonstrable facts relative to these conditions indicate that they are chemical reactions fundamentally related.

It is well known that living organisms in general try at times with a surprising degree of success and again with conspicuous failure to cope with changes in their environment and pabulum; of two men transferred from a balanced diet to a deficient diet one will adjust himself to the change and continue to enjoy good health, the other fails to and suffers scurvy or beriberi; of two pathogenic bacteria invading a man under identical conditions, one fails to withstand the unfavorable elements of its environment and dies, the other survives, multiplies and produces infection. In such cases vulnerability depends upon the lack of power to increase or change the chemical activity of certain cells and fluids of the body; immunity results from the possession of such power.

It would, therefore, seem that idiosyncrasy is a manifestation of cellular inability to respond to a need for increased or new chemical activity, that allergy and anaphylaxis are manifestations of imperfect, inadequate response and that active immunity is the result of adequate response to a need for greater or more diversified chemical activity of certain tissue cells.

Much is yet to be learned as to which cells produce antibodies, variations in their response to different antigens and the exact chemical nature of various antigen-antibody reactions. The application to these problems of better methods of studying colloidal changes and hydrogen ion concentration will extend our knowledge of the mechanism of immunity and variations in response of the host to antigens.

Although the extensive studies of Victor Vaughan and Abderhalden have not produced any new technique or working hypothesis that at present can be applied in medical practice, they have disclosed many facts relative to the chemical aspect of bacterial life and the presence of ferments in blood serum that every immunologist and physician should become familiar with.

ANIMAL INOCULATION

Animal inoculations are occasionally helpful in establishing a diagnosis when other means of investigation fail or are inadequate; they are indispensable in attempts to detect and identify the offending organism in infectious diseases of unknown origin and in the development of specific chemical preparations for the alleviation, cure and prevention of diseases that afflict men and brutes; they are essential to the production of specific sera that in diagnosis and treatment curtail immeasurably the sufferings of man and beast.

If one considers the sheep that have been saved from anthrax, the hogs saved from cholera, the horses saved from lockjaw and animals of all sorts saved from hydrophobia, as a direct result of animal inoculations, and compares this with the total suffering inflicted upon dumb animals by man in his effort to arrest disease, the conviction that these activities have lessened the sufferings of brutes is unavoidable.

Before one can procure desired results from animal inoculation tests in the study of bacteriology several things are necessary—familiarity with the appearance and habits of experimental animals in health, the effect of sudden changes of environment upon them, their normal rate of growth, the normal appearance and relations of their internal organs, the diseases that occur spontaneously in these animals and the changes they produce, and finally, the chain of events to be expected when an animal has been inoculated with a particular species of pathogenic bacteria.

Postmortem examinations of experimental animals should be made and recorded with the same care and detail and by the same general technique described in text books on human pathology in which direction will also be found for the removal, fixation and staining of tissue for microscopic examination.

INDEX TO SUBJECTS

Achorion Schonleini, 159
Acid, formation by bacteria, 129
Acids, as germicidal agents, 24
Acne, 201
Acquired Immunity. *See* Immunity.
  definition of, 262
Actinomyces, 8, 204
  cultivation of, 156, 157, 158
  morphology of, 156
  occurrence of, 155, 157
  staining of, 156
Active immunity. *See* Immunity.
Acute anterior poliomyelitis, 169
Aërobe, 6, 53
Aërobic organisms, obligatory, 6
  facultative, 6
Agar for culture media, 43
  blood, 45
  blood smeared, 45
  slants, for cultivation of bacteria, 50
Agglutination reactions, 65, 66, 67, 111, 112, 113
  clinical diagnosis by, in typhoid, 111
    in differentiating various types of pneumococci, 65, 66, 67
  differentiation of various bacterial species by, 65, 66, 67, 113
  group agglutination in, 113
  macroscopic observation of, for determination of titer limit, 112
  macroscopic observation of, for clinical diagnosis, 111, 112
  specificity of, 113
  upon dead bacteria, 112
  upon living bacteria, 111
Agglutinins, 267
  diluted, agglutination reaction with, 112–114
  experimentation with, 65–113
  group, 113
  in agglutination reactions, 111
  in various immune sera, 111
  nature of 113
  production of, in sera of animals, by, 65
    injection of bacteria, 113
  reaction of, 113
  specificity of, 113
Aggressins, 259
  action of, 259
  character of, 259
  occurrence of, 259
  theoretical consideration of, 259
Air, bacteria in. *See* Individual organisms.
Alcohol, 28
  antiseptic properties of, 28
  as fixative in staining, 22
  fermentation by yeasts, 164
  production of, 164
Alkalies, as germicidal agents, 24
Allergy, 271, 274
Amboceptor, 234, 235, 236, 237
  consideration of, 234, 235
  determination of in Wassermann test, 237
  fixation of complement by, 234
  occurrence and properties of, 234, 237
Anaërobe, 6, 53 54
Anaërobic bacteria, 6, 53, 54
  cultivation of, 53, 54
  facultative, 6
  obligatory, 6
  occurrence of, 130, 132, 134, 136
Anaphylaxis, 271
  definition of, 271
  experimentation in, 271, 272, 273
  observations in, 272
  phenomena of, 271
  proteid injection in, 271
  symptoms in, 272
  theoretical consideration concerning, 273
Anilin-gentian violet, 18
Anilin water, 18
Animals, 275
  experimentation, 275
  inoculations in, 177, 275
Anthrax, 139
  causes of, 139, 140
  occurrence of, 140
  vaccine, 222
Anthrax bacillus, entire consideration of, 139–140
Antianthrax serum, 225

Antibodies, 266, 267
  experimentation and facts concerning,
    agglutinins, 65, 66, 67, 111, 112, 113
    amboceptors, 234, 235
    antitoxin, 223
    bacteriolysins, 267
    opsonins, 266
    precipitins, 267
Anticomplementary, 249
Antigen, 243, 247, 248, 249, 256, 257
  definition of, 243
  for Wassermann test, 243, 247, 248, 249
  for other complement fixation tests, 256, 257
Antimeningococcus serum, 225
Antipneumococcus serum, 67
Antiseptic, determining strength of an, 185
Antistreptococcic sera, 224
Antitoxin, diphtheria. *See* Diphtheria antitoxin.
  tetanus. *See* Tetanus antitoxin.
Arnold steam sterilizer, 37
Arthritis, 210
Arthrospores, consideration of, 6
Asiatic cholera. *See* Cholera.
Attenuation of cultures in manufacture of vaccines, 39
Autoclave, details of, for production of steam under pressure, 35, 36, 38
  Trilatt, 30
  use of, in generating gaseous formaldehyde, 30, 31
Avian tuberculosis, bacillus of. *See* Tubercle bacillus.

Bacilli, classification of, 4–7
  colon-typhoid group of, 103–118
  differentiation, by cultural characteristics, 118
    by morphology, 7
Bacillus, aerogenes capsulatus, 132
    cultivation of, 132
    morphology of, 132
    occurrence of, 132
    pathogenicity of, 133
    resistance of, 132
    staining of, 132
  anthracis. *See* Anthrax bacillus.
  Bordet-Gengou, 78
  botulinus, 130
  bulgaricus, 129
  coli communis, consideration of, 103
    cultivation of, 103
Bacillus coli communis, differentiation from other organisms, 118
    distribution of, 103
    isolation of, on special media, 117 (Plate)
    morphology of, 103
    pathogenicity of, 104
    staining of, 103
  diphtheriæ. *See* Diphtheria bacillus.
  dysenteriæ. *See* Dysentery bacillus.
  Eberth Gaffky. *See* Typhoid bacillus.
  enteritidis, characteristics of, 110
  Friedländer. *See* Bacillus mucosus capsulatus.
  Hoffmani, 89
  influenzæ. *See* Influenza bacillus.
  Klebs-Loeffler. *See* Diphtheria bacillus.
  Koch-Weeks, 78
  lactis aerogenes, 129
  lepræ. *See* Leprosy.
  maligni œdematis, 134
  mallei. *See* Glanders.
    consideration of, 147
    cultivation of, 147
    morphology of, 147
    occurrence of, 147
    pathogenicity of, in animals, 148
      in man, 148
    staining of, 147
    toxin of. *See* Mallein.
  Morax and Axenfeld, 79
  mucosus capsulatus, association of with other organisms, 81
    characteristics of, 80
      cultural, 80
      morphological, 80
      pathological, 81
    staining of, 80
  œdematis maligni, 134
  paratyphoid, characteristics of, 109
    differentiation of, from other organisms, 118
      from various types, 118
    occurrence of, 109
    pathogenicity of, 109
  pestis. *See* Plague.
  pneumoniæ. *See* Pneumonia.
  proteus vulgaris, 128
  prodigiosus, 143
  pseudo-diphtheria, 89
  pyocyaneus, cultivation of, 126
    morphology of, 126
    occurrence of, 126
    pathogenicity of, 127

Bacillus pyocyaneus, pigment production of, 126
  staining of, 126
  virulence of, 127
 smegmatis. *See* Smegma bacillus.
 subtilis, 141
 tetani. *See* Tetanus.
 tuberculosis, avian, bovine and human types of. *See* Tubercle bacillus.
 typhi-exanthematici, 169
 typhosus. *See* Typhoid.
 xerosis, 90
Bacteria, 8
 acid formation by, 129
 acid fast stains for, 19
 acid and alcohol fast stains for, 19–20
 aërobic, 6
 agents injurious to. *See* Sterilization and disinfection.
 anaërobic, 6, 53
 as causes of disease, 263
 attenuated or dead bacteria used for immunization. *See* Vaccine.
 biological consideration of. *See* Individual organisms.
 chromogenic, 55, 126, 143
 classification of, 4
  morphological, 4
  staining, 15, 19
 cultivation of, by aërobic methods, 50
  by anaërobic methods, 53, 54
 destruction of. *See* Sterilization and disinfection.
 discovery of, 1
 differentiation of, by cultural methods, 51
  by fermentation, 118, 119
 enzymes produced by, 259, 260
 gas formation by, 52
 gram negative, 16, 17
 gram positive, 16, 17
 indol production by, 52
 isolation of, 54
 life of, 6
 liquefaction of gelatin by. *See* Individual organisms.
 occurrence of, 1
 parasitic, definition of, 7
 pathogenic, definition of, 7
 pigment production by, 55, 126, 143
 propagation of, by fission, 4
 reproduction of, 4
 saprophytic, definition of, 6
 spore formation of, 5, 6, 8
 staining of, 19
Bacteria, structure of, 5–8
 thermal death points of, 39
 virulence of, 260
Bacteriaceæ, 4
Bacteriological examination of blood cultures, 98, 114
 cat-gut, 179
 feces, 99, 116
 fluid, 178
 milk, 176
 quinine, 179
 sputum, 13, 97
 unknown culture, 13, 178
 urine, 98–116
 water, 173
Bacteriology, development and early history of, 1
Bacteriolysins, 267
Bacterium, 4
Beggiatoa, genus, 5
Beggiatoaceæ, 5
Bichloride of mercury, 24, 33
Bile medium, 42
Bismarck brown, 19
Blastomycetes. *See* Yeast.
Blood agar, 45
 media, 45
 serum (Loeffler), 45
 staining of, 21
Bordet-Gengou bacillus, consideration of, 78
Botulism, 130
Bouillon, 41
Bovine tuberculosis, bacillus of. *See* Tubercle bacillus.
Brilliant green, as an antiseptic, 33
Broth used for culture media, 41
 calcium carbonate, 42
 glycerin, 41
 meat extract, 41
 meat infusion, 41
 sugar, 41

Calcium carbonate broth, 42
Calumette's ophthalmo test, 218
Capsule, staining of, in bacteria, 21
 surrounding of bacteria by a, 5
Carbol-fuchsin, 20
Carbolic acid, as disinfectant, 28, 33
Carbolic acid coefficient, 185
Carbuncles, 202
Carrel-Dakin solution, 25
Cell receptors, various forms as explained in Ehrlich's theory, 269
Chancre, examination of, 152, 208

Chancre, treponema pallidum in a, 152
Chlamydobacteriaceæ, 4
Chloramine as an antiseptic, 28–33
Chlorinated lime as a disinfectant, 25
Chlorine as a disinfectant, 25–33
Cholera asiaticæ, diagnosis of, 123
  discovery of, 2
  epidemics of, 122
  findings of, 123
  spirillum of, consideration of, 122
Chromogenic bacteria, 55
Cladothrices, 155
Classification of bacteria, 4
Clearing of media, 49
Coagulation of milk by bacteria. *See* Individual organisms.
Coccaceæ, 4
Cocci, 7
Coley's fluid, 221
Colon bacillus. *See* Bacillus coli communis.
Colon-typhoid, differentiation, media for, 119
Colonies, counting of, on plates of media, 53, 175
Comma bacillus. *See* Spirillum choleræ asiaticæ.
Complement, consideration of, 234, 238
  definition of, 234
  standardization of, for use in Wassermann test, 238
Complement fixation, action in, 226
  in serum reactions, 226
  in various diseases, 256
  Wassermann test for, 226
Conjunctiva, 205
Conradi-Drigalski medium, 44
Counting of bacteria, 175
Cover-glasses, 11
Crenothrix, 5
Cultivation of bacteria, aërobically, 50–53
  anaërobically, 53, 54
Culture media, 40–54
  filtration of, 48
  formulas, 41
  technique and mode of preparation and sterilization of all, 41–46
  titration of, for standard work, 47, 48
Culture technique, 51

Dakin-Carrel solution, 25
Death point, thermal, for bacteria, 39
Destruction of bacteria, by chemicals, 24–33
  by physical agents, 35–39
Differential staining for bacteria. *See* under Staining
Diphtheria antitoxin, discovery of, 2
  production of, 223
  pseudo-globulins of, 223
  standardization of, 223
  unit of, 224
Diphtheria bacillus, bacillus similar to, 90
  biological consideration of, 82, 83, 84
  cultivation of, 84
  discovery of, 2
  examination for, in mouth, 86, 87
  occurrence of, 82
  pathogenicity of, 85
  resistance of, 84
  staining of, 82
  toxin, properties of, 85
  varieties of, 83, 84
Diplococci, 7
  diplococcus lanceolatus. *See* Diplococcus pneumoniæ.
  diplococcus pneumoniæ, 63
    characteristics of, 63
    cultivation of, 64
    differentiation of, from streptococcus, 64
    morphology of, 63
    pathogenicity of, 64
    resistance of, 64
    staining of, 64
    types of, 65
Diseases of unknown causation, 169
Disinfectants, chemical, 23
  gaseous, 30
  physical, 35
Disinfection, technique of. *See* Sterilization.
Dorsett egg medium, 45
Ducrey's bacillus, 208
Dysentery bacilli, biological consideration of, 119
  characteristics of, 119
  morphology of, 119
  pathogenicity of various types of, 120
  products of, 120
  varieties of, 119

Ear, diseases of the, 205
Eberth, discovery of typhoid, 2
Edema, malignant, bacillus of. *See* Malignant bacillus.
Egg, for preparation of medium, 45
  for use in culture media, 49
Eggs, technique for examination of, 183
Ehrlich's immunity theory, 269
Endo's medium, 43
  for colon-typhoid differentiation, 117

Endospores, 8
Endotoxins, 259
Enzymes, 259, 260
Eosin, preparation of aqueous solution of, 16
Erysipelas, 202. Also *see* under Streptococcus.
Ethylhydrocuprein hydrochloride, 33
Eusol, solution of, as an antiseptic, 28, 33
Eye, diseases of the, 264

Facultative aërobes, 6
  anaërobes, 6
Farcy. *See* Glanders.
Feces, bacteriological examination of, 116
  disinfection of, 25
Fermentation tests, for colon-typhoid differentiation, 118
  in water, 174
Fish tuberculosis, bacillus of. *See* Tubercle bacillus.
Fixation of complement, 227
Flagella, special staining methods, 20
  structure of, 5
Flavine, as an antiseptic, 29, 33
Formaldehyde, as a disinfectant, 29
  entire consideration of all common methods in vogue for the generation of gaseous solution of, 30
Fractional sterilization, 36
Frambœsia, 203
Friedländer bacillus. *See* Bacillus mucosus capsulatus.
Fuchsin, 15
Fungi, mould. *See* under Hyphomycetes.
Furunculosis, 202
Fusiform bacilli, of Vincent's angina, 91

Gabbet's stain for tubercle bacillus, 19, 20
Gäertner, discovery of bacillus enteritidis, 110
Gangrene, 171
Gas formed by bacteria, 52
Gelatin medium, 43
  liquefaction of, by bacteria. *See* Individual organisms.
Gentian-violet, 16
Glanders, bacteriological diagnosis of, 148, 202
  bacillus. *See* Bacillus mallei.
Glycerin, use of, in media, 41
Gonococcus, complement fixation test of the, 256
  cultivation of, 71
  examination for, 72
  infection of, in man, 73
  morphology of, 71
Gonococcus, pathogenicity of, 72
  resistance of, 72
  staining of, 71
  vaccine of, 212
Gonorrhea, 73
Gram negative bacteria, 16, 17
Gram positive bacteria, 16, 17
Gram's method of staining and differentiation of bacteria by, 16
Group agglutination, 113

Haffkine's vaccine, 222
Halogens as disinfectants, 25
"Hanging drop," method of preparation, 13
Heat, different kinds of, in use for sterilization purposes, 35
Hemolysis, 240
Higher bacteria, the, 155
Hiss' serum medium, 42
Hydrophobia. *See* Rabies.
Hypersusceptibility. *See* Anaphylaxis.
Hyphomycetes, classification of, 162
  conditions favorable to growth, 161
  diseases caused by, favus, 159
    ringworm, 159
  morphology, 161
  varieties of, 162

Immunity, acquired, 262
  definition of, 259
  Ehrlich's theory of, 269
  Metchnikoff theory, 268
  natural, 264
  phagocytic theory of, 265
  side-chain theory of, 269
Impetigo contagiosa, 203
Indol, production of, 52
  tests for, 52
Infection, consideration and definition of, 263
Influenza bacillus, 77
  bacteria related to, 78
  biology of, 77
  isolation of, 77
  morphology of, 77
  pathogenicity of, 77
  staining of, 77
Inoculation of media, 51
Iodoform as disinfectant, 29
Iodine and iodine tetrachloride as disinfectants, 25, 33
Isolation of bacteria, 54

Jenner's discovery of immunization in smallpox, 1, 171

Ketchup, examination of, 179
Klebs bacillus. *See* Diphtheria bacillus.
Koch's old tuberculin, 214
Kock-Weeks bacillus, 78

Lactose media, 41
  agar, 41
  bouillon, 41
  litmus agar, 41
Leprosy, 204
  bacillus of, 101
  cultivation of, 101
  morphology of, 101
  occurrence of, 101
  pathogenicity of, 101
  relation of, to tubercle bacillus, 101
  staining of, 101
Leucocytes in milk, 177
Light, effect of, on tubercle bacillus, 96
Lime, chloride of, as a disinfectant, 25
Litmus milk, 41
Lobar pneumonia, 65
Loeffler's blood serum media, 45
  methylene blue, 15
Lungs, diseases of the, 206
Lupus, 203
Lysins, 267
Lysol, 29

MacConkey's bile salt media, 42
Madura foot, 204
Malachite green as an antiseptic, 33
  media, 44
Malignant edema, bacillus of, 134
  consideration of, 134
  cultivation of, 134
  morphology of, 134
  pathogenicity of, 134
  staining of, 134
Mallein, 148
Malta fever, 125
Mastoiditis, 206
Measles, 171
Meat extract bouillon, 41
  preparation of, 41
Meat infusion bouillon, 41
  preparation of, 41
Meningitis, microörganisms causing, 68
Meningococcus, 68
  cultivation of, 68
  early observation of, 68
  morphology of, 68
  pathogenicity of, 69
  serum, 70
Meningococcus, viability of, 69
Metacresol as a disinfectant, 28
Methylene blue, Loeffler's, 15
Micrococci, 7
Micrococcus catarrhalis, consideration of, 74
  intracellularis, meningitidis. *See* Meningococcus.
  melitensis, consideration of, 125
  tetragenus, consideration of, 75
Microörganisms, classification of, 4
  discovery of, 1
  parasitic, 7
  pathogenic, 7
  saprophytic, 7
Microscope, dark-field, 11
  early observations, 9
  present study of bacteria by aid of the, 9, 10, 11
Microsporon audouini, 159
  furfur, 150
Migula's classification of bacteria, 4
Milk, animal inoculation tests in, 177
  as a culture media, 41
  bacteria in, 176
  bacteriological examination of, 176
  diseases caused by, 176
  leucocytes in, 177
  plating test in examination of, 176
Monilia, 167
Moro test, 218
Motility of bacteria, 4–8
Moulds. *See* Hyphomycetes.
Mucor, 162
Mycomycetes, 162

Negri bodies, in rabies, 219
Neisser's method of staining diphtheria bacillus, 18
"New Tuberculin" (Koch), 214
"New Tuberculin," bacillary emulsion, 214
Nose, diseases of the, 206

Obligatory aërobes, 6
  anaërobes, 6
"Old Tuberculin" (Koch), 214
Opsonic index, 2, 265
  test, Wright's, entire consideration of, 265
Opsonins, 264
Orthocresol, as a disinfectant, 28
Otitis media, 205
Oxygen, as a disinfectant, 32

Pappenheim's solution, in use for staining tubercle bacillus, 20

Paracresol, as a disinfectant, 28
Parasites, bacterial, 7
Paratyphoid bacillus, 109
Passive immunity. *See* Immunity.
Pathogenic bacteria, 7
Penicillium, 162
Peroxide of hydrogen, as a disinfectant, 24
Petri dish plates, 50
Petroff's media, 46
    modifications of, 46
Pfeiffer's phenomenon, 123, 264
Phagolysis, 264
Phenol, as a disinfectant, 28, 33
Phenol coefficient test, 185
Physiological normal salt solution, 227
Pigment formation by bacteria, 55, 126, 143
Plague, bacillus of, biology of, 144
    cultivation of, 145
    morphology of, 144
    occurrence of, 144
    pathogenicity of, 145
    resistance of, 145
    staining of, 144
Planococcus, 4
Planosarcina, 4
Plating, 52
Pneumobacillus. *See* Bacillus mucosus capsulatus.
Pneumococcus. *See* Diplococcus pneumoniæ.
Poisons, bacterial. *See* Enzymes.
Polar bodies, special stains for. *See* Neisser's method, under staining.
Poliomyelitis, acute anterior, 169
Potassium dichromate, as a disinfectant, 24
    permanganate, as a disinfectant, 24
Potato media, 46
Precipitins, 267
Preparation of specimens for examination, 11
Pseudo-diphtheria bacillus, 89
Purpura hemorrhagica, 203
Pus, bacteriological examination of, 118
Pus cells and leucocytes in milk, 117
Pyocyanin, 126
Pyrogallic acid, use of, in cultivation of anaërobic bacteria, 53, 54

Rabies, diagnosis of, 218
    negri bodies in central nervous system in cases of, 219
    occurrence of, 218
    pathology of, 219
    treatment of cases with injections of attenuated virus fixe, 220
    virulence and preparation of virus, 220
Receptors, 269
Red blood cells, for Wassermann and other complement fixation tests, 227
Reducing power of bacteria, 52
Relapsing fever, spirochæta, 145
Reproduction of bacteria, 4
Ringworm, 159

Saccharose media, 41
Saccharomycetes. *See* Yeasts and yeast cells.
Salt, normal physiological, solution, 227
Saprophytes, 6
Saprophytic, definition of, 6
Sarcina, 4, 7, 75
Scarlet fever, 171
Schizomycetes, 8
Sclavo's serum. *See* Antianthrax serum.
Sera, therapeutic, 223–225
Serum media, Hiss, 42
    Loeffler's blood, 45
Serum reactions, technique of, agglutination tests, macroscopic, 111
        microscopic, 111
        Widal, 111
    complement fixation, 226
        gonococcus, 256
        other, 256
        Wassermann, 226
Schick test, 88
Side-chain theory, 269
Skin diseases, 201
Slides, 11
Smallpox, etiological factor of, 171
    immunization in, 171
    occurrence of, 171
    treatment of, by virus, 221
Smegma bacillus, 101
    cultivation of, 101
    differentiation from tubercle by staining, 102
    occurrence of, 101
Sodium citrate solution, 227
Solutions, stock, for staining bacteria, 15
Spinal fluid, examination of, 70, 98, 249
Spirilla, 8
Spirillaceæ, 4
Spirillum, description of, 8
    choleræ asiaticæ. *See* Cholera.
    of Vincent, 91
Spirochætæ, dentium, 154
    icterohemorrhaga, 170
    obermeyeri, 145
    of relapsing fever, 145

Spirochætæ, pallida, cultivation, 151
    demonstration of, in smears, by Giemsa's stain, 150
        in tissue, by Levaditi's method, 150
    morphology of, 150
    occurrence of, in syphilis, 150
    staining of, 150, 151
  pertenius, 154
  refringens, 154
  Vincent's, 91
Spores, varieties of, 5, 6, 8
    arthrospores, 6
        bacterial, 5, 6, 8
        endo or true, 8
Sporotrichum Schenkii, 168
Sputum, collection of, 97
  examination of, 97
  staining in examination of, 97
Staining, 15
  acid fast bacteria, 19
    and alcohol fast bacteria, 19, 20
  blood cell, 21
  Gabbet's method of, for tubercle bacillus, 19, 20
  Gram's method of, 16
  method of, for capsules, 21
    flagella, 20
    spores, 21
  Neisser's method, for diphtheria bacillus, 18
  Pappenheim's method of, for tubercle bacillus, 19, 20
  preparation of solutions for, 15–22
Standardization of, amboceptor for Wassermann test, 237
  antigen for Wassermann test, 248
  antitoxin, diphtheria, 224
    tetanus, 224
  complement for Wassermann test, 238
Staphylococci, 7, 55
Staphylococcus, 55
  epidermis albus, 55
  pyogenes albus, 55
    aureus, 55
    citreus, 55
    cultural characteristics, 56
    morphology of, 55
    occurrence of, 55
    pathogenicity of, 57
    resistance of, 57
    toxic products of, 57
    vaccine of, 212
    virulence of, 57
Steam in sterilization, 36, 37, 38
Sterilization, 23–39
    chemical, 23
    fractional, 36
    pharmaceutical, 32, 34, 35
    thorough consideration of, 23–39
Sterilizer, Arnold's steam, 37
    hot air, 35
Streptococci, 4–7, 59, 170
Streptococcus pyogenes, 59
    classification, 59
    differentiation from other organisms, 61, 62
    morphology of, 59
    occurrence of, 59
    pathogenicity of, 60
    resistance of, 60
    staining of, 59
    toxic products of, 60
Streptothrices, 4, 155
Streptothrix maduræ, 158
Syphilis, 203
    microörganism of. *See* Spirochæta pallida.

Test, complement fixation, 226
    Wassermann, 226
    Widal, 111
Tetanus antitoxin, 224
        production of, 224
        standardization of, 224
        unit of, 224
    bacillus, characteristics of, 136
        cultivation of, 136
        morphology of, 136
        occurrence of, 136
        pathogenicity of, 137
        resistance of, 137
        spores of, 136
        toxin of, 137
Tetrads, 7
Thermal death points, 39
Titration of culture media, 47, 48
Torulæ, 164
Toxin, extracellular, 260
    intracellular, 259
Trichomycetes. *See* Chlamydobacteriaceæ.
Trichophyton, 159
Tricresol, 28
Trillat autoclave, 30
Tubercle bacillus, avian, 99
    bovine, 99
    ichthic, 99
    organisms related to, 101, 102
        of leprosy, 101

Tubercle bacillus, organisms related to, smegmatis, 101
Tubercle bacillus, biological consideration of, 94
  cultivation of, 95
  examination for, 20, 94, 95
    by Gabbet's method, 20
    by Pappenheim's method, 94
    by Ziehl-Nielson method, 95
  in feces, 99
  in milk, 99
  in sputum, 97
  in urine, 98
  method of staining for the, 20, 94, 95
  morphology of, 94
  pathogenicity of, 96
  resistance of, 95
  staining of, 94
  toxic products of, 96
Tuberculin, 213
Tuberculosis, mode of infection, 94
  tuberculin as a diagnostic reagent in, 217, 218
    in the treatment of, 217
Typhoid bacillus, biological consideration, 106
  cultivation of, 106
  differentiation of, from other organisms, 117, 118
  discovery of, 2
  examination and isolation of, 114, 116, 118
    in blood during disease, 114
    in feces, 116
    in sputum, 118
    in urine, 116
  morphology of, 106
  pathogenicity of, 107
  staining of, 106
  toxic products of, 106
  vaccine of, 212
Typhoid fever, diagnosis of, by agglutinins in blood serum, 111
  by blood culture, 114
  Widal test, 111

Unit, for amboceptor, 237
  antigen, 244
  antitoxin, 224
  complement, 238
  diphtheria antitoxin, 224
Unit, tetanus antitoxin, 224
Urethritis, 208
Urine, bacteriological examination of, 98, 116

Vaccine, autogenous, 211
  polyvalent, 211
  sensitized, 213
  stock, 211
    definition of, 211
    production of, 211
Vaginitis, 208
Variola. *See* Smallpox.
Vincent's angina, spirochæta of, 91
  cultivation of, 91
  consideration of, 91
Virulence, 260
Virus fixe, 220
Von Pirquet test, 218
Von Ruck's tuberculus, 215

Wassermann test for the diagnosis of syphilis, 226
  amboceptor, 234, 237
  antigen for, 247
    standardization of, 248
  complement in, 234, 238
  controls in the, 244
  preparation for, 239
  reading of, 246
  technique of, 245
  value of, 250
Water, bacteria in, 173
    number of colonies of, 173
    pathogenic, cholera, 175
      typhoid, 106
  bacteriological examination of, 173–175
Welch bacillus, 132
Wertheim's media for cultivation of gonococcus, 71

Yaws, 154
Yeast cells, consideration of, 164
  cultivation of, 164
  pathogenic, 165
  reproduction by budding, 164
  saprophytic, 164
  varieties of, 165
Yellow fever, 171

Ziehl-Nielson's method for staining tubercle bacillus, 95

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